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CHANGES IN THE HISTOCHEMICAL APPEARANCES OF CHOLINESTERASE IN A MIXED PERIPHERAL NERVE FOLLOWING NERVE SECTION AND COMPRESSION INJURY

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Received for publication May 22, 1957

THE histochemical examination of the distribution of cholinesterase in the normal sciatic nerve of the guinea-pig shows that it is concentrated mainly in the axons and in the neurilemmal sheaths, and a small amount is present in the connective tissue between the nerve fibres (Snell, 1957). The present research was designed to investigate histochemically the effect of nerve section and compression injury on the distribution of cholinesterase in a mixed peripheral nerve and to ascertain the exact position of the enzyme in the degenerating and regenerating nerve fibres.

MATERIALS AND METHODS

Guinea-pigs were used for the investigation and these were divided into three groups: (*a*) In 17 animals the right sciatic nerve was cut in mid-thigh and a 1 cm. length removed. (*b*) In a further 17 animals the right sciatic nerve was cut but the two ends were immediately approximated by means of a single silk suture. (*c*) In 6 animals the right sciatic nerve was firmly compressed by artery forceps.

Animals from each group were killed by a blow on the back of the neck at intervals over a period of six weeks following the operation. The first animals were killed 1, 2, 3 and 6 days after the operation and thereafter the remainder were killed at intervals of approximately 4 days. The injured sciatic nerve was removed from each animal and at the same time the normal nerve was removed from the other leg to serve as a control. The nerves were fixed in 10 per cent formol saline for 15 hr. Longitudinal frozen sections 30 μ thick were then cut and washed in distilled water for 30 min. Sections were then processed using a modification of the technique described by Koelle and Friedenwald (1949). In order to hasten the precipitation of copper thiocholine and so reduce diffusion artefacts the pH of the substrate mixtures was lowered to 4·2. Sections were incubated with the substrate mixtures at 37° for 48, 72 and 96 hr. The cholinesterase appearances in the sciatic nerves were assessed by studying the concentration and distribution of the copper sulphide precipitate which formed after the addition of ammonium sulphide. In order to distinguish between the cholinesterase group of enzymes and the other esterases a number of the nerve sections were treated with incubating solutions also containing 0·00003 M eserine. In these investigations no distinction was made between true and pseudo-cholinesterase.

RESULTS

Cholinesterase appearances in control nerves

The histochemical appearances of cholinesterase in all the control sections which had been incubated for the same length of time were practically identical. The cholinesterase was seen to be concentrated mainly in the axons and in the neurilemmal sheaths, and a small amount was present in the connective tissue between the nerve fibres (Fig. 1).

Cholinesterase appearances in experimental nerves

In all groups of animals the concentration of cholinesterase in the proximal portion of the nerve remained normal (Fig. 2), while in the portion distal to the site of the lesion the cholinesterase became reduced in amount as degeneration proceeded (Fig. 3).

As soon as regeneration began the nerve fibrils growing out of the proximal stump were found to contain a concentration of cholinesterase much greater than that seen in the normal nerve (Fig. 4–6). Similar appearances were noted in the nerve fibrils growing along the peripheral neurilemmal tubes in the nerves which had been cut and sutured (Fig. 7) and in those which had been damaged by compression (Fig. 8).

DISCUSSION

Couteaux and Nachmansohn (1938) have shown by chemical analysis that in the peripheral part of the cut sciatic nerve in the guinea-pig the concentration

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EXPLANATION OF PLATES

In all photomicrographs, cholinesterase appears black and has been stained histochemically without counterstain, except in Fig. 7.

FIG. 1.—Transverse section (50 μ thick) of normal nerve. Shows cholinesterase to be concentrated mainly in the axons and in the neurilemmal sheaths. × 1540.

FIG. 2.—Longitudinal section of proximal uninjured portion of nerve 20 days after nerve section. Shows nerve to contain a moderate amount of cholinesterase. × 95.

FIG. 3.—Longitudinal section of distal portion of nerve 20 days after nerve section. Shows droplets of broken down myelin and Schwann cells. The concentration of cholinesterase is very much reduced. × 70.

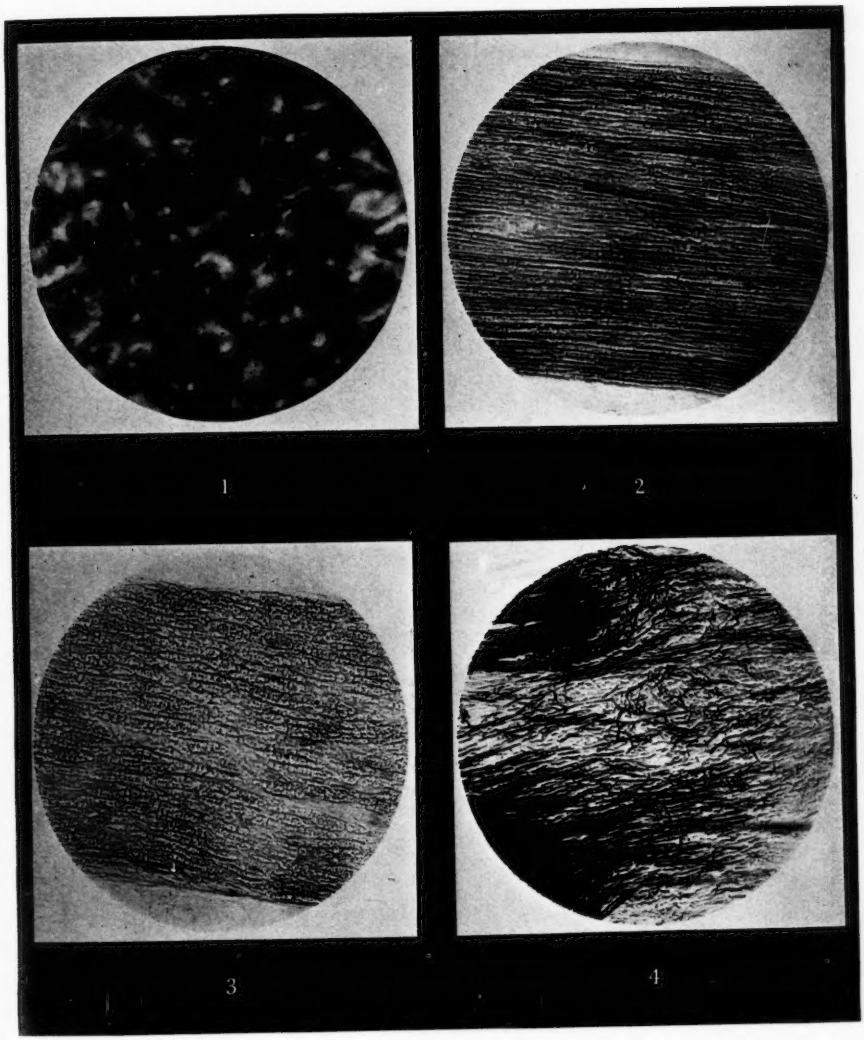
FIG. 4.—Longitudinal section of neuroma of proximal portion of nerve 34 days after nerve section. Shows large number of nerve fibrils growing out of proximal stump containing a very high concentration of cholinesterase. × 120.

FIG. 5.—Longitudinal section of proximal portion of nerve 20 days after nerve section and suture. Shows large number of nerve fibrils growing out of the proximal stump containing a very high concentration of cholinesterase. × 115.

FIG. 6.—Longitudinal section of neuroma 20 days after nerve section and suture. Shows large number of nerve fibrils growing through thread suture. The nerve fibrils contain a very high concentration of cholinesterase. × 90. A = nerve fibrils; B = thread suture.

FIG. 7.—Longitudinal section of distal portion of nerve 20 days after nerve section and suture. Shows nerve fibrils growing down amongst the neurilemmal cells and myelin droplets. The nerve fibrils contain a high concentration of cholinesterase. Counterstained with Sudan III. × 90.

FIG. 8.—Longitudinal section of crushed nerve 2 days after injury. Shows large number of outgrowing nerve fibrils in the crushed area containing a very high concentration of cholinesterase. × 613.



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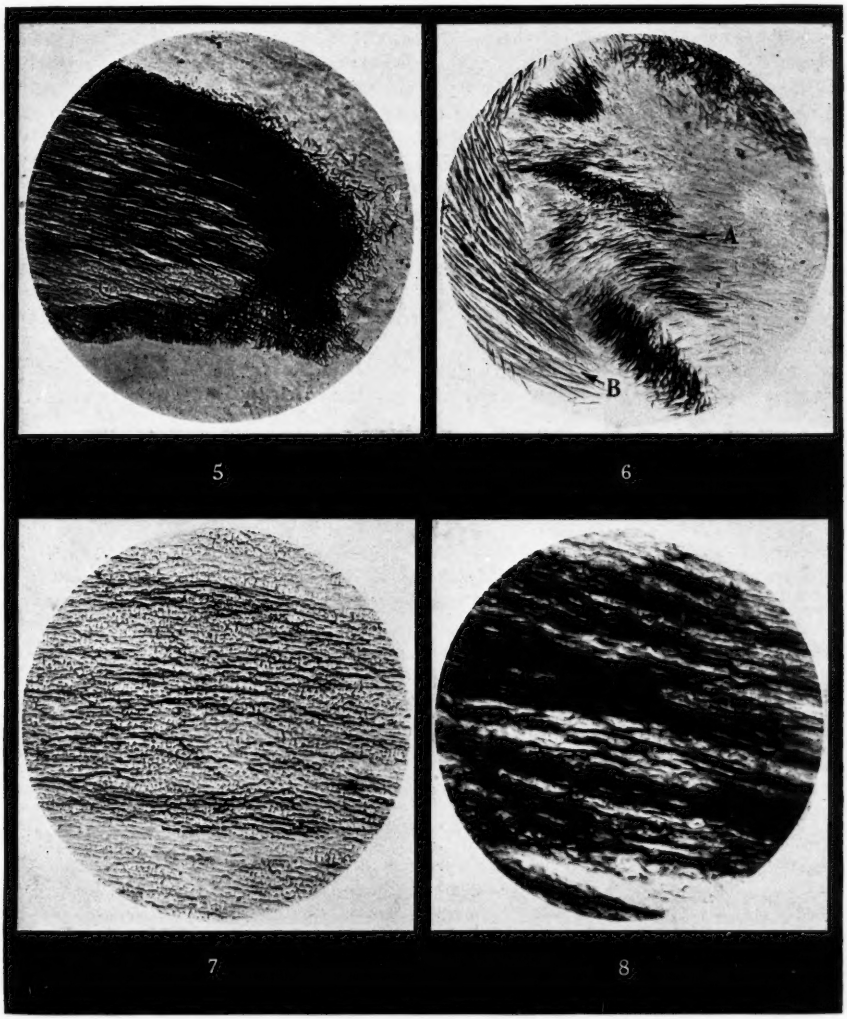

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of cholinesterase after two weeks decreases by about 30 per cent, whereas in the neuroma formed at the central end of the cut nerve the concentration increases to a value which is twice as high as in the normal nerve.

Sawyer (1946) has shown by chemical analysis that during the first few days after section of the sciatic nerve in the same species there is a 60 per cent loss of the true cholinesterase in the peripheral portion of the cut nerve. Pseudocholinesterase activity was found to be unaffected by the operation. Analysis of the neuroma at the central end of the cut nerve showed that the cholinesterase hydrolysing power was three times as great as that in the control, and the rise was due almost entirely to the increase in true cholinesterase. Sawyer, however, was unable to determine by the method used whether the cholinesterase present in a degenerating or regenerating nerve was situated in the macrophages, fibroblasts, Schwann cells or in the outgrowing nerve fibrils.

The results of the present investigation show that the fall in concentration of cholinesterase in the peripheral stump during Wallerian degeneration is almost certainly due to the disintegration of the axis cylinders, since only a small amount of residual cholinesterase was seen in the peripheral neurilemmal tubes. The very high concentration of cholinesterase seen in the nerve fibrils growing out of the proximal stump would account for the high concentration of this enzyme found by chemical analysis in the neuroma by Couteaux and Nachmansohn (1938) and by Sawyer (1946). Furthermore the findings of Sawyer would suggest that the type of cholinesterase present in the nerve fibrils is true cholinesterase.

Cholinesterase is known to hydrolyse acetylcholine but the rôle it plays in the process of nerve conduction is still a matter of conjecture. Nachmansohn (1948) believes that acetylcholine is involved not only in synaptic transmission but also in the conduction of the nerve impulse along the nerve fibre. This theory has, however, been widely criticised and evidence against this has been reviewed by Eccles (1948) and Gerard (1950).

We are thus still far from a full understanding of the many biochemical and physical processes involved in normal nerve activity, and until we have solved these fundamental problems, we are unlikely to understand the functional rôle of cholinesterase in the nerve fibrils growing out of the proximal stump in a regenerating nerve, or why it is present in such high concentration.

SUMMARY

The effect of nerve section and compression injury on the histochemical appearances of cholinesterase in the sciatic nerve has been studied in guinea-pigs.

As degeneration proceeded the concentration of cholinesterase in the proximal portion of the nerve was found to remain normal, while in the portion distal to the site of the lesion the cholinesterase became reduced in amount. When regeneration began the nerve fibrils growing out of the proximal stump were found to contain a concentration of cholinesterase much greater than that seen in the normal nerve.

The relationship of these findings to the results produced by chemical analysis by other workers is discussed.

For this research I gratefully acknowledge the technical assistance of Miss J. M. Burke. My thanks are also due to Mr. S. Hogwood for the photomicrographs.

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BACTERAEMIA IN IRRADIATED MICE

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SEVERE bacteraemia has been found frequently in mice dying from X-irradiation (Miller, Hammond and Tompkins, 1950). From studies on the effect of radiation dose on the frequency of bacteraemia (Gonshery, Marston and Smith, 1953), infection appears to be heavy in animals suffering from the bone marrow syndrome which causes death during the second week after total body irradiation with moderate X-ray doses. In the intestinal type of death resulting from larger doses, bacteraemia was incidentally found. Since in both the intestinal and the bone marrow syndrome invading organisms were members of the enteric flora, they were presumed to have passed the lining of the intestinal tract which is known to be sensitive to irradiation. The presence of enteric bacteria in cultures of mesenteric lymph nodes suggested that entrance into the circulation might be through the lymphatic system (Gordon, Ruml, Hahne and Miller, 1955). Coliform bacteria and *Proteus* were the predominating organisms in irradiation bacteraemia; a varying incidence of *Pseudomonas* and *Streptococcus faecalis* was reported. In most of the blood specimens one type of organism was found and it was shown that bacteraemia may last for several days except in *Pseudomonas* infections which were rapidly fatal (Hammond, Tompkins and Miller, 1954).

In our experience, *Pseudomonas* bacteraemia can be prevented by adequate sanitation which includes disinfection of drinking bottles and removal of carriers from animal stocks. Blood cultures from irradiated mice belonging to a *Pseudomonas*-controlled colony gave results which differed from those reported by other authors. In the present investigation data were collected on blood and lymph node infections in mice irradiated with X-ray doses varying between 675 and 1200 r.

MATERIALS AND METHODS

Animals

CBA mice between 8 and 12 weeks old and weighing 18–26 g. were used. This strain was bred by brother × sister mating exclusively. Stock animals were kept in groups of 20–30 mice in aluminium pans. During the week preceding the irradiation fresh water in disinfected bottles was provided daily; fresh sterilized sawdust bedding was given every other day. Before irradiation, samples from faeces and drinking bottles were cultivated for the presence of *Pseudomonas*. Animals from positive pans were not used. After irradiation the mice were housed individually in glass boxes, with water and sawdust as before. A commercial cubed food was supplied *ad lib.* Details of the procedures leading to the elimination of *Pseudomonas* infection are to be published (Wensinck, van Bekkum and Renaud, 1957).

Irradiation

The mice were irradiated in groups of 10 animals in a circular perspex cage. The X-ray factors were: 200 kv, 20mA, filtration 1·5 mm. Cu, H.V.L.: 1·8 mm. Cu, distance to target

50 cm., dose rate 45 r./min. The dosage was measured with a Baldwin substandard ionization chamber placed on the bottom of the perspex box. Maximal backscatter was employed. The irradiation dose varied between 675 and 1200 r. The relation between X-ray dose and mortality in CBA mice has been reported by van Bekkum (1956) to whom we are indebted for placing the irradiated animals at our disposal. The LD_{100} was 675 r.

Methods of culture

Blood agar, salt agar and Endo medium were used for the isolation of micro-organisms from the heart's blood and from lymph nodes. Blood agar was prepared by adding 5 per cent sterile defibrinated sheep's blood to molten peptone agar consisting of 1 per cent (w/v) Difco peptone, 0·3 per cent Difco meat extract, 0·5 per cent NaCl, 1 per cent glucose and 1·5 per cent Difco agar. Salt agar was prepared by adding 6·5 per cent NaCl to 1 per cent lactose agar containing 1 per cent peptone, 0·3 per cent meat extract and 1·5 per cent agar. Endo medium was prepared from Difco dehydrated Endo agar.

One drop of blood, obtained by heart puncture, was spread on the media which were incubated for 48 hr. at 37°. Lymph node suspensions were prepared in saline by grinding the tissue with sand in a mortar. The paired cervical, submaxillary and parotid nodes and the major mesenteric lymph node were examined. One drop of the suspension was plated on the solid media; suspensions were also cultivated in 5 per cent sheep's blood peptone broth, from which subcultures were made on the solid media.

The micro-organisms isolated were streptococci and Gram-negative bacteria. The combined blood, salt and Endo agar media were useful for preliminary identification. Characteristic enteric organisms, *e.g.*, *Str. faecalis* and *Bacterium coli* were easily identified on salt and Endo agar. *Paracolobactrum* was identified by the appearance of lactose-fermenting colonies on prolonged incubation of Endo media. The streptococci which did not grow on salt and Endo medium and *Str. faecalis* were further studied by methods described in the following paper (Wensinck and Renaud, 1957). They include Lancefield grouping on HCl extracts from 20 per cent serum broth cultures with the precipitating sera A–N (Burroughs, Wellcome and Co.).

RESULTS

Irradiation with 675 *r.*

The mortality distribution in animals irradiated with this dose shows a peak on the 11th day after irradiation. Bacteriological examination of blood samples from mice killed in the second week after irradiation showed that bacteraemia was always present (Table I). In most of the cases hundreds of colonies were found on plates inoculated with one drop of heart's blood. Streptococcus bacteraemias were predominating; mixed infections were exceptional. The streptococci could be differentiated into two groups, *viz.*, *Str. faecalis* and minute streptococci. The latter grew on blood media with the formation of pin-point colonies surrounded by a small zone of α-haemolysis which was well developed after incubation for 48 hr. Subcultures in blood broth showed the presence of long entangled chains of small streptococci. Classification with the aid of fermentation and other tests was not decisive. Lancefield grouping tests, however, showed specific reactions with group G serum. Most of the minute streptococcus strains isolated from irradiated mice were tested and all were found to belong to group G. There were no cross reactions with the grouping sera A-N. Characteristics of the group G streptococcus will be given in a separate paper. This micro-organism was found to be a common constituent of the flora of the upper respiratory tract; in some cases, group G streptococci were also found in the intestine.

Group G streptococci formed the majority of streptococcal isolates in the 675 r. series. Of 82 positive blood cultures obtained from animals sacrificed from 4–11 days after irradiation with 675 r. (animals of Table I included) 73 per

cent were group G streptococci and 8·5 per cent were *Str. faecalis*. It is clear from these results that the characteristic enteric organisms are of minor importance in the bone marrow syndrome bacteraemias in CBA mice.

TABLE I.—*Bacteraemia in Irradiated Mice Killed* 8–11 *Days after Irradiation.*

	Dose	
	675 r.	800 r.
Number of mice	52	30
Bacteraemia present	52	30
Group G streptococci	40	9
Str. faecalis	4	2
Bact. coli	4	1
Mixed infections	4*	18†

* *Bact. coli* + streptococci.
† Group G streptococci + *Bact. coli* or *Str. faecalis.*

Irradiation with 800 *r.*

The animals were killed 8–10 days after irradiation and all were found to have a bacteraemia (Table I). Mixed infections, in which large numbers of group G streptococci and enteric organisms were found together, were much more frequent than in the 675 r. series.

Irradiation with 950 *r.*

Two groups of animals were irradiated with different results (Table II). The divergence of the survival time which could not be attributed to sex or housing conditions was thought to be due to a small difference in radio-sensitivity. It is known that there is a rather sudden change from the bone marrow syndrome (survival period 8–12 days) to the intestinal type of death (survival period 4–6 days) on increasing the X-ray dose. It is likely that the dose of 950 r. has been in this transitional dose region. Striking differences were also found between the results of blood cultures in these two groups. Bacteraemia was always found in animals dying during the second week after irradiation (Table III Group A).

TABLE II.—*Mortality Distribution after Irradiation with* 950 *r.*

	Deaths* on post-irradiation day							
Group	4	5	6	7	8	9	10	11
A	—	1	—	3	1	26	15	3
B	33	11	1	—	3	2	—	—

* Animals killed when moribund included.

TABLE III.—*Bacteraemia in Mice Irradiated with* 950 *r.*

	Group A Table II	Group B Table II
Number of mice	49	50
Bacteraemia present	48	13
Group G streptococci	11	—
Bact. coli	15	11
Str. faecalis	3	—
Paracolobactrum	—	1
Mixed infections	19*	1†

* Group G streptococci + *Bact. coli* or *Str. faecalis.*
† Group G streptococci + *Bact. coli.*

Mixed infections were frequent and in a third of the samples enteric organisms were found exclusively. It is clear from these results that enteric bacteraemia is much more important in this group than in animals irradiated with 800 r. In the early deaths occurring after irradiation with 950 r. (Table III Group B) bacteraemia was occasionally found. *Bact. coli* predominates in this group.

Irradiation with 1200 *r.*

Most of the blood samples from animals killed on the 3rd and 4th day after irradiation were sterile; enteric organisms were found in the blood of many animals on the 5th and 6th day (Table IV). The number of organisms however, was small; in many cases less than 10 colonies developed on plates inoculated with one drop of heart's blood.

TABLE IV.—*Bacteraemia in Mice Irradiated with* 1200 *r.*

Post-irradiation day	Number of animals	Bacteraemia present
3	8*	0
4	32*	8
5	14†	10
6	3†	3

* Animals were in rather good condition when killed.
† Animals found dead or killed when moribund.

Classification of irradiation bacteraemias

The above results suggest that bacteraemias in irradiated CBA mice may be arranged in three groups according to the radiation dose. In the bone marrow syndrome bacteraemias (675 r.), group G streptococci predominate and give rise to an overwhelming infection. Intestinal deaths (950–1200 r.) are associated with a much less severe bacteraemia which is exclusively caused by enteric organisms. The intermediate group (800–950 r.) is characterized by mixed infections in which both group G streptococci and enteric organisms are invasive.

The distribution of G streptococci, which were commonly found in the upper respiratory tract of CBA mice, suggests that the respiratory epithelium and the cervical lymphatic system may be involved in the propagation of group G streptococcus infections. It seemed therefore of interest to cultivate the lymph nodes of irradiated animals at various stages of the bone marrow syndrome. The results were compared with those obtained in animals irradiated with 1200 r.

Infection of lymph nodes

Two groups of animals were used which were irradiated with 750 r. and 1200 r. respectively. The heart's blood and lymph nodes from animals killed on various days after the irradiation were cultivated. Occasionally, difficulties were encountered in obtaining sufficient material from the nodes, particularly the cervical ones, which were greatly reduced after the irradiation; in these cases, the tissue surrounding the nodes was also dissected out. The results of the lymph node cultures from animals irradiated with 750 r. are given in Tables V (pre-bacteraemic period) and VI (bacteraemic animals). As appears from the data in Table V most of the samples were sterile. In positive cultures, a very small number of group G streptococci was found. Table VI shows that these organisms could be

isolated from all lymph nodes cultivated when blood counts exceeded 50 colonies per drop of heart's blood ; lymph nodes were frequently found to be sterile at the initial stage of bacteraemia. Enteric organisms were not found in any specimen. These findings indicate that infection of lymph nodes in this series of animals was merely a result of bacteraemia.

TABLE V.—*Results of Lymph Node Cultures from Mice Irradiated with 750 r. and Killed in the Pre-bacteraemic Stage*

Days after irradiation	Number of animals	Lymph node			
		Parotid	Submaxillary	Cervical	Mesenteric
1	5	—	—	—	—
3	4	—	—	—	—
4	11	—	1+	—	2+
5	9	—	1+	—	—
6	6	—	1+	—	—
7	1	—	—	—	—

— = all cultures negative ; + = group G streptococcus found in culture ; numeral = number of positive samples.
Blood cultures were negative in all of these animals.

TABLE VI.—*Results of Lymph Node Cultures from Mice Irradiated with 750 r. in which Streptococcus Bacteraemia was Present*

Number of group G streptococci in blood specimen	Number of animals	Lymph node			
		Parotid	Submaxillary	Cervical	Mesenteric
<10	5	1+	2+	2+	1+
10–50	4	—	3+	2+	—
>50	6	6+	6+	6+	6+

— = all cultures negative ; + = group G streptococcus found in culture ; numeral = number of positive cultures.

The observations in animals irradiated with 1200 r., which are represented in Table VII, make it clear that mesenteric lymph nodes may be heavily infected in the period which precedes death. In most of the positive specimens, the numbers of organisms amounted to thousands per lymph node. *Bact. coli* was the predominating organism ; *Str. faecalis* and *Paracolobactrum* were found in some specimens only. Group G streptococci were not isolated from the lymph nodes.

TABLE VII.—*Results of Lymph Node Cultures from Mice Irradiated with 1200 r. and with Negative Blood Cultures*

Days after irradiation	Number of animals	Mesenteric lymph node	Pooled parotid, cervical and submaxillary lymph nodes
3	8	—	—
4	20	10+	—

— = all cultures negative ; + = enteric organisms found in cultures ; numeral = number of positive cultures.

DISCUSSION

The occurrence of large numbers of group G streptococci in the heart's blood of irradiated mice is the major point of difference between the results presented

in this paper and the data on irradiation bacteraemia reported in the literature. This streptococcus was found in the majority of the bone marrow syndrome bacteraemias (675 r.) and was never isolated from blood specimens of mice suffering from the intestinal syndrome (950–1200 r.) in which enteric organisms invaded the circulation. With increasing X-ray dose, therefore, the relative frequency of the streptococcus bacteraemia decreases. Gonshery *et al.* (1953) observed a similar effect. The streptococci isolated by these authors and by Miller *et al.* (1950) were characterized by α-haemolysis and were thought to be enterococci. We found however that *Str. faecalis* isolated from mice is indifferent on blood media. It may therefore be supposed that the α-streptococci isolated by Miller and Gonshery were murine strains of group G streptococci, which show marked greening.

Bacteraemia accompanying the intestinal type of death may be well explained by the current hypothesis of its enteric origin. Intestinal organisms invade the circulation, mesenteric lymph nodes are found to be infected with these organisms and denudation of the mucosa of the small intestine is a characteristic feature of this syndrome.

The fact that lymph nodes from mice irradiated with 750 r. are sterile in the pre-bacteraemic period indicates that the origin of bone marrow syndrome bacteraemia is different. Group G streptococci are not pathogenic for mice and their number in the respiratory tract is very small compared with other streptococci. It is therefore suggested that specific factors are involved in the mechanism which promotes streptococcal invasion in the irradiated animal.

SUMMARY

Large numbers of group G streptococci were found in the heart's blood of mice irradiated with a total body dose of 675 r. This organism predominates in the bone marrow syndrome bacteraemia ; occasionally, bacteraemia was found to be caused by enteric bacteria. The intestinal syndrome (950–1200 r.) was accompanied by invasion of enteric organisms which probably pass into the circulation by way of the lymphatic system ; large numbers of these organisms were found in the mesenteric lymph nodes.

In animals irradiated with X-ray doses varying from 800–950 r. mixed infections were frequent ; group G streptococci and enteric organisms were found together in the blood.

The lymph nodes of animals suffering from the bone marrow syndrome were sterile in the pre-bacteraemic period. It is suggested that different factors are involved in the mechanisms leading to streptococcal and enteric bacteraemia.

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MURINE GROUP G STREPTOCOCCI

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GROUP G streptococci have been frequently found in the blood of irradiated CBA mice suffering from the bone marrow syndrome (Wensinck and Renaud, 1957). Reference was made to investigations showing that this streptococcus is a common inhabitant of the respiratory tract of CBA mice. The data concerning the occurrence of streptococci in various parts of the body are presented in this paper together with the characteristics of the murine group G streptococcus. The existence of a non-haemolytic variant of group G is also reported.

METHODS

Isolation of streptococci

CBA mice were killed with coal-gas ; suspensions in saline were prepared from the nasal septum, larynx, trachea, lung, tongue, small intestine and rectum. Blood agar and salt agar were inoculated with one drop of the suspension. Streptococcus colonies were picked off into blood broth.

Methods of identification

The extracts for grouping were prepared from 18 hr. cultures in serum broth according to the method of Lancefield (1933) ; commercial sera (Burroughs, Wellcome and Co.) were used. Fermentation tests were carried out in a medium devised by de Moor (see following section) to which carbohydrates (0·5 per cent) were added. The production of gelatinase was tested by growing the strains on de Moor's medium to which agar (1·5 per cent) and gelatine (0·4 per cent) had been added. Plates were incubated for 4 days at 37° and flooded with $HgCl_2$ solution ($HgCl_2$, 15 g.; conc. HCl, 20 ml.; H_2O, 100 ml.) ; clear zones around colonies indicate gelatinase activity (Smith, Gordon and Clark, 1946). Hydrolysis of hippurate was tested in de Moor's medium with sodium hippurate (1 per cent) added. Cultures were centrifuged after incubation for 4 days at 37° and 12 per cent ferric chloride solution in 2 per cent HCl was added to the supernatant. Ammonia was detected by adding Nessler's reagent to 4-days-old cultures in de Moor's medium to which L-arginine HCl (0·3 per cent) had been added. Growth in the presence of bile was tested on 1 and 4 per cent Bacto ox-gall blood plates which were incubated for 48 hr. at 37°. Strong reduction was tested on cultures growing in skimmed milk with 0·1 per cent of methylene blue (B.D.H.) added. Growth at 45° was tested in 20 per cent serum broth. In the study of pathogenicity 0·2 ml. of 18 hr. cultures in blood broth was injected intraperitoneally into 5 mice. The animals were observed for a week.

Culture media

Blood broth was prepared by adding 5 per cent defibrinated sheep's blood to peptone broth consisting of 1 per cent Difco peptone, 0·3 per cent Difco meat extract, 0·5 per cent NaCl and 1 per cent glucose. The pH was adjusted to 7·3 before sterilization. The medium was solidified by adding 1·5 per cent Difco agar. Salt agar was prepared by dissolving 6·5 per cent NaCl in peptone broth containing 1·5 per cent agar ; the medium was sterilized for 20 min. at 120° and 1 per cent lactose was added after the sterilization. The streptococcus medium of de Moor was prepared by dissolving 1·5 per cent Difco casitone (or B.B.L. trypticase), 0·5 per cent Difco yeast extract, 0·02 per cent L-cystine, 0·01 per cent sodium sulphite,

0·25 per cent NaCl and 0·2 per cent disodium phosphate in distilled water ; 50 ml. of a 0·2 per cent solution of phenol red were added per l. The medium was sterilized for 15 min. at 115°.

RESULTS

Characteristics of the Murine Group G Streptococcus

Minute colonies surrounded by a small and sharply defined zone of α-haemolysis develop on blood agar after incubation for 24 hr. A granular deposit is formed in blood broth and consists of small Gram-positive cocci arranged in very long entangled chains. The same type of growth is observed in serum broth. No growth occurs on the ordinary media in the absence of blood or serum. A great range of carbohydrates is fermented (Table I). When first encountered in irradiation bacteraemia, the strains were thought to belong to the viridans group. However grouping tests showed strong and specific reactions with the group G serum. The morphological and biochemical properties of some 20 strains isolated from both normal and irradiated mice were uniform.

TABLE I.—*Fermentation Reactions of Group G Streptococci*

	Action on red blood cells		
	α-Haemolysis	Indifferent	β-Haemolysis
Glucose	A	A	A
Lactose	A	A	A
Saccharose	A	A	A
Raffinose	A	—	—
Mannitol	A	—	—
Aesculin	A	A	—
Trehalose	A	A	A
Sorbitol	A	—	—
Inulin	A	—	—
Arabinose	A	—	—
Salicin	A	A	A

A = production of acid. — = no acid produced.
α-haemolytic strains (more than 20) were isolated from mice, indifferent strains (2) from porcine and human sources and the β-haemolytic strain from a human carrier.

β-Haemolysis was not induced by anaerobiosis and the appearance of the colonies was not influenced by the type of blood used in an agar medium in which horse, human, mouse, rabbit or sheep blood had been incorporated. The murine strains are therefore not β-haemolytic. The results of a survey of various organs for the presence of streptococci which are presented in Table II show that group G streptococci are frequently found in the larynx and may occasionally be isolated from other parts of the body. Their number was always very small. So far, this streptococcus has only been found in mice. Table I shows the significant differences between the fermentation reactions of the murine group G streptococcus and those of a β-haemolytic strain isolated from a human carrier. The morphology is also different ; the β-haemolytic cocci are arranged in short chains and are larger than the long-chain murine cocci.

Another group G variety has been isolated from human and animal sources by de Moor (personal communication). This variant forms indifferent colonies on blood agar but the fermentation tests agree well with those of the β-haemolytic large colony form (Table I). There was also close resemblance in morphology

between this indifferent variety and the β-haemolytic strains. All group G strains were non-pathogenic for mice and failed to grow at 45° or in the presence of 6·5 per cent NaCl, 0·1 per cent methylene blue or 1 or 4 per cent Difco ox-gall. Milk was curdled by many strains. Tests for gelatinase were negative. Hippuricase was formed by the murine strains but not by the β-haemolytic and indifferent strains. Tests for arginase activity were positive in all strains.

Other Streptococcus Species Isolated from CBA Mice

Streptococcus faecalis and *Streptococcus equinus* were the commonest streptococci. *Str. faecalis* was confined to the intestinal canal and was never isolated from cultures of the tongue. All strains were indifferent on blood agar and formed transparent colourless colonies on salt agar. *Str. equinus* was the predominating species in the respiratory tract and was isolated in large numbers from the tongue. The strains were characterized by the inability to ferment lactose and by delayed hydrolysis of aesculin. Marked α-haemolysis was produced on blood agar.

Table II shows the distribution of the streptococcus species in CBA mice.

TABLE II.—*Distribution of Streptococci in CBA Mice*

Organ	Number of samples	Species isolated*		
		Str. equinus	*Str. faecalis*	Group G
Nose	10	0	0	2
Larynx	15	15	0	13
Trachea	10	10	0	4
Lung	10	0	0	0
Tongue	25	25	0	0
Small intestine	10	5	10	3
Rectum	10	0	10	0

* Numeral = number of positive samples.

DISCUSSION

This survey of the streptococcal flora of CBA mice was made in connection with the finding that group G streptococci frequently invade the circulation in irradiated mice suffering from the bone marrow syndrome (Wensinck and Renaud, 1957). It appears from the distribution pattern that this streptococcus is a common inhabitant of the respiratory tract of normal mice. After irradiation of the mice the distribution of group G streptococci remains unchanged up to the onset of bacteraemia. It is suggested therefore that the respiratory epithelium may be the portal of entry for group G streptococci invading the irradiated animal. In a study of tongue lesions in irradiated mice (Vos, Wensinck and van Bekkum, unpublished) necrotic foci were observed in the larynx which was occasionally removed together with the caudal part of the tongue. The number of larynx specimens studied so far is not large enough to justify the assumption of a relationship between radiation damage to the respiratory epithelium and streptococcal invasion.

The presence of the group G polysaccharide in the α-haemolytic and indifferent strains described in this paper indicates that the association between the type of polysaccharide and haemolytic power is variable. Comparable patterns of variation have been reported for other Lancefield groups, *e.g.*, group H

(Porterfield, 1950) and group L (McLean, 1955). The α-haemolytic strains belonging to these groups generally showed physiological characteristics which closely resemble those of the corresponding β-haemolytic strains and are therefore considered to be group variants. The same applies to the indifferent strains of group G. The biochemical activity of the α-haemolytic group G strains however widely diverges from the group characteristics (*cf.*, Simmons and Keogh, 1940; Wahl and Meyer, 1956). It is suggested therefore that this streptococcus belongs to a quite distinct species which, so far, has only been found in mice. Clearly, the group G polysaccharide is shared by a heterogeneous group of organisms, *viz.*, the large colony and minute β-haemolytic forms, the indifferent variant and the α-haemolytic murine streptococcus.

SUMMARY

Group G streptococci were found to be common inhabitants of the respiratory tract of CBA mice. This finding is discussed with regard to data on irradiation bacteraemia reported before.

The morphological and biochemical characteristics of the α-haemolytic murine group G streptococci are markedly different from those of β-haemolytic strains.

An indifferent variant is also reported to belong to group G. This variant closely resembles the β-haemolytic strains. The group G polysaccharide is shared by a heterogeneous group of organisms, *viz.*, β-haemolytic, indifferent and α-haemolytic streptococci.

We wish to thank Dr. C. E. de Moor for generously sending us the indifferent group G variety and for helpful advice.

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THE *IN VITRO* DIFFUSION OF ANTIBIOTICS THROUGH FIBRIN MEMBRANES

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IT is well known that the host-parasite relationship in certain infections due to various micro-organisms is such that there is little stimulation of antibacterial immunity. This applies in particular where the organisms are confined within the initial site of infection by the deposition of fibrin barriers in the connective tissues. Previous work by Hughes (1948) has demonstrated that membranes prepared from human fibrin have a selective effect with regard to the passage of various dyes, toxins and proteins. Comparatively little attention has been paid, however, to the problem of what effect fibrin barriers have on the diffusion of antibiotics. One of the few studies is that of Weinstein, Daikos and Perrin (1951) who showed that penicillin is able to diffuse freely into fibrin clots implanted subcutaneously in rabbits.

The present investigation was undertaken in an attempt to determine whether fibrin membranes would exert a selective action on the passage of a number of antibiotics *in vitro* since the rational use of antibiotic therapy in infections characterized by fibrin deposition will obviously be determined to some extent by the ability of the drugs to penetrate the fibrin barrier.

MATERIALS AND METHODS

Antibiotic stock solutions.—Stock solutions of crystalline penicillin G (20,000 units/ml.), chloramphenicol (1,000 μg./ml.) and chlortetracycline (1,000 μg./ml.) were prepared in sterile 0·85 per cent saline. Solutions of erythromycin and spiramycin (500 μg./ml. of each) were prepared by dissolving 250 mg. of the pure substance in 0·5 ml. of ethanol and then diluting with sterile 0·85 per cent saline to the required concentration.

Stock antibiotic solutions were stored in the deep-freeze without loss of potency during the period of the investigation. For use stock solutions were diluted with 0·85 per cent saline on the day required.

Fibrin membranes.—Preliminary studies were made to determine the most satisfactory method of producing a standard type of fibrin membrane. The method finally adopted made use of a straight sterile sintered glass filter tube (porosity No. 1, internal diameter 10 mm., thickness of filter disc 3 mm.). The side arms were heated and bent to an angle of 45° midway between the filter disc and the ends of the tubes. A series of such tubes was prepared.

To make the fibrin membrane the filter tube was clamped vertically to a retort stand and the filter disc was saturated with fresh sterile human plasma from a donor. Immediately saturation was complete, usually with a volume of approximately 0·15 ml. of plasma, a drop of sterile commercial thrombin solution was added to cause clotting. The tubes were left for 30 min. and were then placed horizontally in the clamp. Simultaneously, 5·0 ml. of antibiotic test solution were added to the left-hand limb of the tube and 5·0 ml. of sterile saline (pH 7·0) to the right-hand limb. In this way unequal pressures on either side of the membrane were avoided and the volumes used were sufficient to cover both sides of the membrane completely.

At varying intervals of time 0·25 ml. volumes were removed from the right-hand limb and stored in the deep-freeze for subsequent assay of antibiotic content. A corresponding volume was also removed from the left-hand limb and discarded in order to equalize the pressures on either side of the membranes. A final check was also made on the potency of the antibiotic test solutions at the end of each period of observation.

Assay of test fluids. All assay procedures were carried out using serial doubling dilutions of the test fluid in digest broth in 0·5 ml. amounts. The test organism used for each was a stock culture of *Staphylococcus pyogenes* (var. *aureus*, N.C.T.C. 6571). The inoculum consisted of an 0·02 ml. amount of a 8-hr. digest broth culture. Results were read after 24 and 48 hr. of incubation.

Testing of fibrin membranes.—Intact fibrin membranes will retain the dye Congo red (Hughes, 1948), and this was made use of in determining the intactness of the membranes. A 0·1 per cent solution of Congo red was substituted for the fluid in the right-hand limb of the tubes, after the test period was over, and left for 6 hr. Any diffusion of the dye during this period was taken to indicate a possible defect in the membrane. With a little practice it was found that intact membranes could be produced without much difficulty.

RESULTS

The assay results obtained with the various antibiotics are detailed in the Table and represent the average findings of repeated observations made at varying intervals of time. Using Congo red as an indicator of the state of the membrane it was found that after about 60 hr. there was passage of the dye. After 48 hr. the membranes were intact but it was considered that observations beyond this period might be unreliable.

TABLE.—*Antibiotic Concentrations on the Opposite Side of a Fibrin Barrier at Different Time Intervals*

	Antibiotic				
Time (hr.)	Penicillin 2000 units/ml. (units/ml.)	Chlor-amphenicol 200 μg./ml. (μg./ml.)	Chlor-tetracycline 200 μg./ml. (μg./ml.)	Erythro-mycin 100 μg./ml. (μg./ml.)	Spiramycin 100 μg./ml. (μg./ml.)
1	300	0	0	0	0
2	400	0	2	0	0
3	800	1	4	0	0
4	900	2	5	0	0
5	1000	5	8	1	0
8	1000	8	10	1·5	0·5
10	1000	8	12	2·0	1·0
12	1000	10	18	3·0	1·5
16	1000	12	26	4·0	2·0
20	1000	16	28	4·0	2 5
24	1000	16	30	5·0	2·5
36	1000	18	35	6·5	3·0
48	1000	20	40	7·0	3·5
Theoretical final concentration assuming equilibrium	1000	100	100	50	50

The diffusibility of penicillin through this type of membrane was far superior to that of the other antibiotics tested, complete equilibrium with the test solution being attained in about 5 hr. A series of observations with penicillin concentrations ranging from 100 to 1,000 units/ml. showed no difference in the time taken for equilibrium to be reached. These observations are in agreement with the *in vivo*

findings of Weinstein *et al.* (1951) who showed rapid diffusion of penicillin into tissue-implanted clots in rabbits.

With erythromycin and spiramycin the maximum concentrations attained on the opposite side of the fibrin membranes were approximately 1/7 and 1/14 of the theoretical final concentrations assuming free diffusion across the membranes with equilibrium. Both chloramphenicol and chlortetracycline appeared to diffuse more freely but again the final concentrations after 48 hr. represent only 1/5 and 2/5 respectively of the theoretical equilibrium concentrations. Experiments carried out with oxytetracycline and tetracycline yielded results similar to those given by chlortetracycline.

Observations were made on the diffusion of penicillin, chloramphenicol and chlortetracycline at 4·0° and at 37·0°. With chloramphenicol and chlortetracycline there was slightly more rapid diffusion at 37·0° during the first twelve hr. than there was at room temperature but the final concentrations after 48 hr. were practically identical with those at room temperature. The rates of diffusion at room temperature and at 4·0° appeared to be similar. For penicillin there was no apparent difference in the rate of diffusion at all temperatures.

DISCUSSION

The rôle of fibrin barriers in inflammatory foci has received attention mainly from the point of view of the efficiency of the barrier in preventing organisms and toxins from passing into the general circulation. According to Menkin (1940), fibrin deposited in lymphatic channels and tissue spaces constitutes an important barrier to such passage. Hughes (1948) and Dubos (1955) have pointed out that fibrin acts in a selective manner allowing the passage of certain toxins but retaining micro-organisms although allowing the passage of leucocytes. Day (1954) found that the perfusion of mouse connective tissue with plasma in which clotting was in actual process led to a decrease in permeability.

Whether or not an antibiotic will pass through a tissue fibrin barrier is of considerable importance since complete eradication of an inflammatory focus may be largely dependent on adequate access of the drug to the site of infection. The present results show that under *in vitro* conditions fibrin barriers may exert a selective action on the passage of certain antibiotics. The choice of a suitable antibiotic may thus depend on the nature of the lesion in addition to the activity of the drug on the causative organism.

The diffusion of an antibiotic through fibrin is almost certainly a complex matter involving factors other than the pore size of the membrane and the concentration of the antibiotic. Some of the *in vivo* factors which may influence such diffusion are difficult of reproduction in an *in vitro* study. The constant biochemical changes taking place locally in the tissues which are the seat of infection may considerably alter the rate and amount of diffusion. In addition the pore size of the membrane both *in vitro* and *in vivo* will tend to alter as the result of the spiral configuration which the fibrin molecules tend to assume during the process of clot retraction. Amongst the other factors involved, diffusion may be influenced by chemical changes taking place between the antibiotic and the amino or carboxyl groups of the fibrin molecules. In addition the degree of binding of the antibiotic to the protein of the tissue fluids may affect the amount of diffusion. With regard to the local biochemical environment of the lesion alterations in the pH of the

fluid within the area of inflammation may be of importance. Dubos (1955), for example, has shown that in tissues which are the site of a staphylococcal infection there may be a considerable rise in pH associated with increased glycolytic activity and a fall in the tissue glucose content.

In the experimental model described here we have made use of a standard thickness of membrane but diffusion through an *in vivo* barrier will obviously be influenced by variations in the thickness of such membranes. In coagulase-producing staphylococcal infections very considerable amounts of fibrin may be deposited. Fibrinolysis, however, will also occur to a certain extent *in vivo*. Where streptococci produce large amounts of streptokinase the deposition of fibrin may be minimal. Part of the reason for the ultimate deterioration of the type of membrane made use of in this *in vitro* study is probably fibrinolysis due to the spontaneous conversion of plasminogen, adsorbed on the fibrin during the clotting process, to plasmin.

In the early stages of the formation of an inflammatory focus the increased tissue pressure around the lesion may be sufficient to allow the passage of an antibiotic which will not diffuse readily once the fibrin barrier is formed. With the formation of the inflammatory exudate the pressures inside and outside the lesion tend to reach equilibrium and the subsequent passage of antibiotic will then depend mainly on diffusion processes. Treatment, however, will seldom be instituted at the stage where the tissue pressures outside the lesion are above those in the lesion itself. Consequently we have ignored the effect of filtration pressures in these experiments in favour of the results of diffusion. It is not of course possible to draw any analogy between the arrangement of fibrin strands in tissue spaces and those in the interstices of a sintered glass filter since too many variable factors exist *in vivo* which cannot be allowed for in the experimental model. It is interesting, however, that Hughes (1948) found that the filtration of serum through fibrin membranes *in vitro* gave rise to a filtrate very similar in composition to that of an inflammatory exudate.

The findings for penicillin agree with those of Nathanson and Liebhold (1946) as well as those of Weinstein *et al.* (1951). The former workers also showed that penicillin would diffuse freely into agar. Masonyi, Held and Kocsan (1949), on the other hand, reported that penicillin would only penetrate fibrin layers greater than 3 mm. with difficulty. Werner, Knight and MacDermott (1954) also reported that penicillin, streptomycin, chloramphenicol and chlortetracycline diffused readily into agar discs *in vitro* and *in vivo*. It seems probable, however, that these findings have little reference to the problem of fibrin barriers in view of the known selective effect of the latter.

SUMMARY

The diffusion of five antibiotics through fibrin membranes has been investigated *in vitro*.

Penicillin diffuses rapidly through such a membrane attaining equilibrium on both sides of the barrier in five hours. The passage of chloramphenicol and chlortetracycline across such a membrane was considerably less, resulting in about one-fifth and two-fifths respectively of the theoretical equilibrium concentrations, after a period of 48 hours. Two other antibiotics, erythromycin and spiramycin, diffused across the membrane with still greater difficulty.

The relationship of these findings to *in vivo* conditions is uncertain since a number of factors are concerned which cannot be allowed for in the experimental model.

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OBSERVATIONS ON THE DELAY IN GASTRIC EMPTYING AFTER X-IRRADIATION IN THE RAT AND THE EFFECT OF ADRENALECTOMY UPON IT

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A DELAY in the emptying of the stomach is one of the manifestations of the radiation syndrome in the rat and, as the rat is unable to vomit, may be analogous to the nausea and vomiting seen in other species. The phenomenon was first noticed by Ely and Ross (1947) in experiments with neutrons and has since been studied as an effect of X-rays. Goodman, Lewis and Schuck (1952) evolved a method for studying gastro-intestinal transit using Evans blue and applied it to rats which had been subjected to whole body irradiations of 450 r. of X-rays. Billings, Bennett and Burlingame (1954), using the same dose, studied the effect of whole and part body irradiation by taking radiographs at various time intervals after forced feeding with a barium sulphate-water mixture. Swift, Taketa and Bond (1955) have weighed the stomach contents after various doses of irradiation and Fenton and Dickson (1954) using the same method have shown that mice too develop a delay in gastric emptying after irradiation.

Serial radiography following a barium meal appeared to be a suitable method to confirm and extend the findings of previous workers. A simple method has been used in which the rat takes the opaque meal voluntarily. This has the advantage of allowing observations to be made on the same rat at different time intervals and does away with the forced feeding as used by Billings *et al.* which might itself provoke abnormalities of stomach function. The effects of adrenalectomy were also investigated in order to test the relation of the phenomena to the stress syndrome.

METHODS

Male rats of an inbred albino strain 3 to 6 months old were used in groups of up to 5 litter mates. The animals were allocated to the various irradiations and other procedures in a random manner and at least one of each litter was used as a control.

Feeding.—The barium preparation used was a commercial product of approximately 2 parts of barium sulphate and 1 part malted milk (Horlicks Shadow Meal). In order to accustom the rats to the taste of the preparation and to eat from a metal spatula they were offered each day, and soon eagerly accepted, a thick paste made from the same malted milk used in the opaque meal (*i.e.*, Horlicks). When fully used to this procedure they were starved overnight, *i.e.*, during their normal feeding time, in order to prepare their alimentary tract for radiography. The next day each rat was fed with 5 g. of the barium sulphate-malted milk mixture made into a thick paste with 13 drops of water. The fixed consistency of the opaque meal was important in order to give as little variation as possible within experimental groups in the rate of passage of the meal and also to provide the rats with material which they could manage to eat with ease.

Radiography.—As soon as possible after feeding and before irradiation, control radiographs were taken to give a base line for the amount of filling of the stomach. The appropriate

dose of irradiation was given immediately afterwards and each animal, including the control was radiographed at ½, 1, 1½, 2, 2½, 3, 4, 5, 6, 7 and 8 hr. after the time of irradiation and occasionally at later times. Radiographs were taken without unduly restraining the animals by using a tunnel-like perspex container. The diameter was such that an adult rat fitted snugly inside and the sides were made opaque so that the rats could not see out except at the far end where air holes were placed. The other end was closed by a piece of perspex made to slide into one of two slots according to the length of the rat, a gap being left to accommodate the tail. While in the container the rats remained remarkably still and in a suitable position for radiography without showing any sign of being frightened. Left lateral and postero-anterior views were taken at each time interval. During each exposure the rats received a dose of approximately 0·05 r. Food was not made available until after the last radiograph was taken so that the barium sulphate should not be diluted.

Dosage.—Whole body doses of 20 r., 30 r., 50 r. and 100 r. were given, the rats being irradiated singly or in pairs in an aluminium box. The radiation factors were: 240 kV, 43 r./min.; 240 kV, 48·8 r./min. or 250 kV, 68 r./min. with a H.V.L. of 1·2 mm. Cu. in each case. The radiation field was uniform to ± 3 per cent. Sham irradiations were carried out on all control animals.

Adrenalectomy experiments

Operations were performed 6 to 14 days before irradiation. Half the animals in each litter, chosen at random, were subjected to bilateral adrenalectomy using the dorsal approach. In the other animals an exactly similar operation was performed except that the adrenals, after being identified, were left *in situ*. All but one of the adrenalectomized animals died within a few weeks of the operation and at autopsy no adrenal remnants were seen. The one exception, a non-irradiated animal, continued to gain weight after the experiment. When it was killed one month later a large part of one adrenal was found *in situ*. The results obtained from this animal were discarded.

Mode of assessing results

A qualitative assessment of the effects of a procedure was usually apparent from comparison with the radiographs of other animals in the same litter but as such assessments are of little use in comparing different doses of irradiation an attempt has been made to produce a semi-quantitative estimate of the stomach contents. By comparing each radiograph with the pre-irradiation control films five stages in the appearances of the stomach were easily distinguished and each radiograph was scored accordingly.

1. Completely full of radio-opaque material (100 per cent).
2. Three quarters full (75 per cent).
3. Half full (50 per cent).
4. One quarter full (25 per cent).
5. A remaining trace (arbitrarily awarded 10 per cent).

An average was then obtained for each time interval in the various experimental groups and graphs were drawn relating degree of emptying to time after irradiation.

A dose response curve for the plain irradiation experiments was obtained by calculating the averages and standard errors of the combined scores from 5 to 8 hr. inclusive after irradiation of the control and irradiated animals.

RESULTS

Dosage experiments

Fig. 1 shows the results obtained for doses of 20 r., 30 r., 50 r. and 100 r. together with those of their litter-mate controls. In the control animals the stomach usually begins to show evidence of emptying between the second and third hr., after which it empties rapidly until only a small amount of opaque material remains. This remnant is then cleared more slowly. With doses of 20 r. and 30 r. the emptying begins a little later and proceeds at a less rapid rate, the change

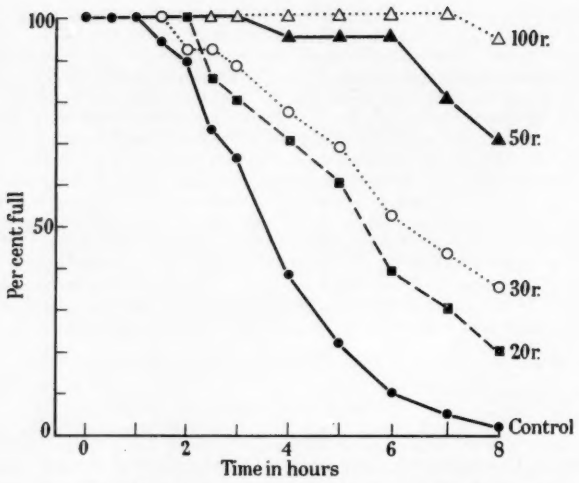

FIG. 1.—The effect of X-irradiation on the pattern of gastric emptying. The curves for the various doses are shown on the graph ; the control is an average of 13 animals, 20 r. is an average of 5, 30 r. of 6 and 100 r. of 4 animals.

being rather more marked at the dose of 30 r. A dose of 50 r. results in a more pronounced delay and usually it is not until 6 hr. after irradiation that any clear evidence of emptying is seen. With a dose of 100 r. very little change in the size of the stomach shadow occurred during the 8 hr. of continuous observation. The dose response relationship (Fig. 2) is linear over the time interval studied for doses up to 50 r.

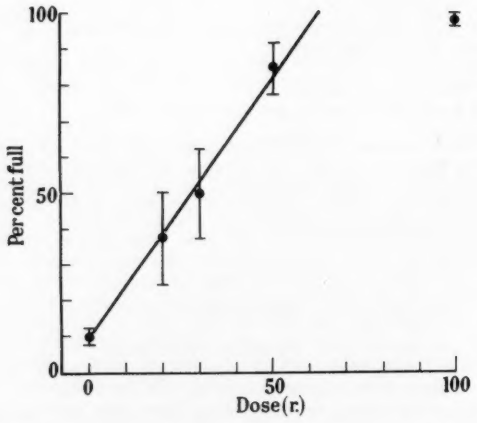

FIG. 2.—Dose-response curve for the delay in gastric emptying after whole body irradiation. Each point represents the averages with standard errors of the degree of fullness of the stomach over the period 5 to 8 hr. in Fig. 1.

Adrenalectomy experiments

It was intended that each litter should provide 2 adrenalectomized and 2 sham-operated rats, 1 of each pair to be irradiated. In spite of both pre-operative and post-operative training it was found more difficult to feed rats which had been operated upon, particularly at the shorter time interval between operation and feeding. This difficulty resulted in 2 animals (1 adrenalectomized and 1 sham-operated) eating so little of the barium mixture that their stomachs were not even three-quarters full in the pre-irradiation radiograph and they were therefore discarded. The results are consequently based on 7 adrenalectomized and 7 sham-operated animals which received 50 r. and 5 adrenalectomized and 6 sham-operated animals which were used as controls.

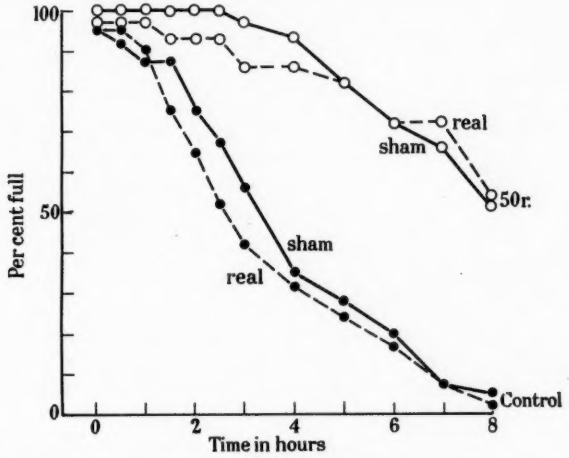

FIG. 3.—The effect of adrenalectomy on the pattern of gastric emptying in control and irradiated animals.

– – ● – – Adrenalectomized animals, no irradiation.
——●—— Sham-operated animals, no irradiation.
– – ○ – – Adrenalectomized animals, 50 r. whole body X-rays.
——○—— Sham-operated animals, 50 r. whole body X-rays.

The average values for these experiments are shown in Fig. 2. The feeding difficulties already referred to are shown by the lower starting point of three of the curves. There is however no significant difference between the rate of stomach emptying of those animals which had had both adrenals removed and those in which only a sham operation had been performed.

DISCUSSION

Post-irradiation gastric dysfunction has been notoriously difficult to assess in man and although recent studies of the duration of the latent period between therapeutic irradiation and the onset of symptoms have allowed a more precise study of the post-irradiation syndrome (Court Brown and Mahler, 1953; 1954;

Court Brown and Abbatt, 1955) there is still a need for animal experiments to assess the effects of possible modifying factors and to attempt to elucidate the exact nature of the phenomenon. Animal experiments are also less likely to be complicated by psychological factors which are known to produce symptoms after sham therapeutic irradiation in man (Court Brown, 1953) and might complicate the study of low doses when nausea is likely to be the only symptom. Care must be taken however to reduce the interference with the animal to a minimum. Part-body irradiation without anaesthesia was attempted in this series of experiments and it was found that in the control animals the necessary restraint frequently gave rise to a gross delay in the stomach emptying. The experiments were therefore abandoned but it was felt that the results obtained demonstrated the importance of self-feeding rather than forced feeding and the value of not restraining the animals during radiography.

Delay in gastric emptying time after X-irradiation in the rat has been reported by several authors (Goodman *et al.*, 1952 ; Billings *et al.*, 1954) but it was not until the paper of Swift *et al.* (1955) that any observations on the lower dose ranges were published. These authors in this section of their investigation confined their observations to weighing the stomach contents at a time interval of 6 hr. after irradiation and found a " border line effect " with 25 r. By using the radiographic technique described in this paper and taking twelve observations over a period of 8 hr. it has been possible to recognize a definite effect with 20 r. in the strain of rats used. Apart from any interest in establishing a low limit for a radiation effect this result also emphasizes the caution necessary in interpreting results either clinical or experimental on stomach movements when frequent periods of screening have been necessary. In the present instance the total radiation dose to each animal, including the controls from all the radiography to the 8 hr. film was about 0·6 r.

Swift *et al.* found a linear relationship between the logarithm of the degree of emptying and the logarithm of the dose at 6 hr. after irradiation. The present approach to the problem using an average of the degree of emptying over 5 to 8 hr. gives a linear relationship using arithmetic scales for doses from 0 to 50 r. of X-rays. The points for 20 r., 30 r. and 50 r. also fit a log. log. linear relationship, but it is considerably steeper than that obtained by Swift *et al.*, due to the greater sensitivity of the method.

The comparison of these experimental results using low doses with the responses in man, as already indicated, is difficult. The most relevant observations are perhaps those on a group of Marshall Islanders who received whole body irradiation lasting about 12 hr. from fall-out following an experimental thermonuclear explosion in which most of those exposed were unaware of the significance of the fall-out. About two-thirds of a group of 64 individuals who received an estimated dose of 175 r. of gamma rays were nauseated and one tenth vomited. One person from a group of 18 who unknowingly received an estimated dose of 69 r. of gamma rays was nauseated. No nausea or vomiting was seen in a group of 28 receiving an estimated dose of 78 r. and a group of 157 receiving an estimated dose of 14 r. of gamma rays (Cronkite, Bond and Durham, 1956).

A delay in emptying of the rat's stomach has been noted in various stress-producing phenomena including severe haemorrhage, traumatic shock, muscle ischaemia and changes in environmental temperature (Swift *et al.*, 1955 ; Whiteley and Green, 1952 ; Geiger and Pinsky, 1956) and it is therefore not surprising that

it has been suggested that the phenomenon is a non-specific response to injury (Swift *et al*). Des Marais and Dugal (1951) have in fact found that an excess of adrenaline is produced on exposure to low temperatures and Geiger and Pinsky have suggested that this is the cause of the delay in gastric emptying with cold which they found. Local production of adrenaline cannot be ruled out as a cause of a delay in gastric emptying in the present experiments but the lack of any change in response to irradiation following adrenalectomy does indicate that the delay is not mediated by either the adrenal cortex or medulla. Other post-irradiation phenomena once thought to be part of a stress reaction have been shown to be due to other causes (Mole, 1956) and similarly the present results indicate that the delay in gastric emptying after X-irradiation is not due to a general adaptation type of response.

SUMMARY

A method is described by which serial radiographs following barium meals may be used to study stomach emptying in the rat. This has been applied to the study of the delay in gastric emptying produced by X-irradiation and doses as low as 20 r. whole body irradiation have been found to produce a delay in gastric emptying. There is a simple arithmetic straight line relationship between dose of irradiation and average degree of emptying for the period 5 to 8 hours after irradiation.

Adrenalectomy neither increases nor decreases the delay in gastric emptying and there is consequently no evidence of the phenomenon being part of the stress response.

It is a pleasure to acknowledge the help I have received during the course of these experiments from the radiographers of this unit, namely Mrs. M. E. D. Lester, Miss J. M. Wheatley and Mr. M. J. Corp. Mrs. Lester also designed the container to keep the rats in position during radiography aud helped in evolving a suitable routine for the training and feeding of the rats.

I also wish to thank Dr. R. H. Mole for his advice and helpful criticism.

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THE HEAT PRODUCTION CONSEQUENT ON INJURY

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FOLLOWING fracture of a long bone, or indeed any severe trauma, there is in man and the rat an increase in the nitrogen content of the urine (mainly as urea), sulphur (mainly as sulphate), phosphorus (as phosphate) and potassium when maintained on a normal diet. The subject is usually in negative balance in these respects during the first few days of the post-injury period and exhibits tissue loss which is of more general than local origin, and in man as much as 6·7 per cent of the body's content of nitrogen may be lost during the first 10 days following injury, even on a normal food intake. The nitrogen to sulphur ratio of the material catabolised is similar to that of muscle. There is also a creatinuria. These observations (Cuthbertson, 1930, 1932; Cuthbertson, McGirr and Robertson, 1939) have been confirmed by many others. The loss of tissue nitrogen can be reduced but not eliminated by the consumption of additional food, but the nitrogen-saving effect observed may be akin to that observed when excess food is consumed by the normal subject.

One of us observed that in man there was a rise in the "basal" consumption of oxygen during this period, and a slight rise in body temperature amounting to not more than 2° F. after injury (Cuthbertson, 1932). One suggestion is that there is an increased demand for energy from tissue sources by the injured animal (Cuthbertson, 1936; Cuthbertson *et al.*, 1939). It seems reasonable to assume that in some way metabolism is raised, presumably in order to facilitate the healing process. But it was obviously necessary to conduct metabolism studies on a more accurate basis than previously available and measure the increase in metabolism consequent on fracture. As one of us (Pullar, 1956, 1957) had constructed a gradient-layer direct calorimeter on the Benzinger Kitzinger principle (Benzinger and Kitzinger, 1949) and capable of taking animals up to 50 lb. (23 kg.) live-weight, it was decided to use this instrument to measure, if present, the rise in heat production following injury of a group of rats fed a diet rich in protein and also to study the effect in others on a practically protein-free regime. The latter treatment was introduced because Munro and Cuthbertson (1943) had observed that the characteristic post-injury rise in urinary nitrogen did not occur in rats on a protein-free diet.

EXPERIMENTAL

The animals used were 3-months-old male hooded rats of the Rowett Institute strain and they were housed as previously described for such experiments (Campbell, Sharp, Boyne and Cuthbertson, 1954). Fracture was the form of trauma used and was induced under ether anaesthesia. A small skin incision was made, the muscle sheaths bluntly separated, and the femur snapped at its mid-point. The wound had one stitch.

In both the experiments nine rats were placed in individual cages inside the calorimeter and their total metabolic rate measured for 22 hr. on each of about 14 consecutive days. Each

day at 10.00 a.m. the animals were removed from the calorimeter, weighed, given fresh food and water, and returned with these to the chamber by 10.30 a.m. The 22-hr. period did not begin until mid-day, by which time the calorimeter had settled. The total metabolic rate for 24 hr. was obtained by proportion from the measurement for 22 hr. The temperature in the calorimeter was 19° ± 0·3° and the relative humidity was about 60 per cent; these conditions were the same as those prevailing in the ordinary rat house of the Institute.

The metabolic rate is expressed in kcal./kg.$^{0·75}$ to exclude the effect of the variation in metabolic rate with weight. It was first measured for 4 days to accustom the rats to the calorimeter and to obtain a steady level. The effect of the short period of ether anaesthesia required for the induction of the trauma was tested on the 5th day. The anaesthesia lasted only a few min. and was without effect on the metabolic rate of the subsequent period following re-entry to the chamber. (It was not considered necessary to check this in the second experiment.) The total metabolic rate was then measured for 22-hr. daily periods for 9 or 10 days following injury. Urine collection was not possible for rats in the calorimeter, because of space considerations, but urinary nitrogen figures were available from other animals which were maintained in the animal house under conditions similar to those existing in the calorimeter.

In the first experiment the animals were fed a diet containing 20 per cent protein (2·9 per cent N) and 3·9 kcal./g., each animal receiving throughout the experiment the amount of food it ate daily before fracture. Refusals were small even on the day of fracture. The intake is recorded in Fig. 1.

In the second experiment another nine rats were fed *ad libitum* on a diet containing 0·1 per cent protein and 4·2 kcal./g. (Campbell and Kosterlitz, 1953). They received this diet from 1 week before the beginning of the metabolism studies. Litter-mates were maintained under similar dietary conditions in the animal house and the urinary nitrogen was determined as in previous experiments (Campbell, *et al.*, 1954). The intake is recorded in Fig. 2.

RESULTS

High protein diet

The total metabolic rate expressed in kcal./kg.$^{0·75}$/day for the average rat maintained on the protein-containing diet was calculated for the 5 days of the immediate pre-operation period and for the 10 days following fracture. The total metabolism of the average rat remained at 145 kcal./kg.$^{0·75}$/day prior to injury and the degree and duration of anaesthesia necessary for the division of the bone was without effect on this. On the 1st day after fracture the heat production remained the same but then rose to a maximum of 152 kcal./kg.$^{0·75}$/day on the 3rd day, which, according to the urinary nitrogen investigations, is the time of the maximum excretion of nitrogen for a rat subjected to this treatment. The metabolic rate gradually declined after the peak and returned to the pre-operation level 9 days after fracture (Fig. 1). This agrees closely with the duration of increased urinary nitrogen. Body weight was maintained and food consumption remained steady (Fig. 1).

Before fracture the metabolic rate of an average rat, whose weight would be 350 g., was 66·1 kcal. and it rose to a peak of 69·0 kcal.

From the nitrogen excretion in the urine of a comparable group on the same diet (Campbell *et al.*, 1954) it was possible to calculate the protein metabolised and to estimate the heat produced in its metabolism. The heat produced consists of the S.D.A. effect, that is, the heat liberated in deamination and the energy cost of excretion of the end products, together with the heat obtained by oxidation of the deaminised amino acids. The magnitude of the S.D.A. effect is subject to a number of influences and can only be estimated. In the calculations the following assumptions are made: (1) 6·25 g. tissue protein contain 1 g. nitrogen; (2) ingestion of 100 kcal. of protein leads to the elimination of 130 kcal., the 30 kcal.

being the S.D.A.; (3) 1 g. of protein on oxidation in the body yields 4·1 kcal., therefore the associated S.D.A. is 1·2 kcal.

The rats used in the N estimations, which weighed 320 g. on average before fracture, were excreting a mean level of 380 mg. urinary N daily prior to the opera-

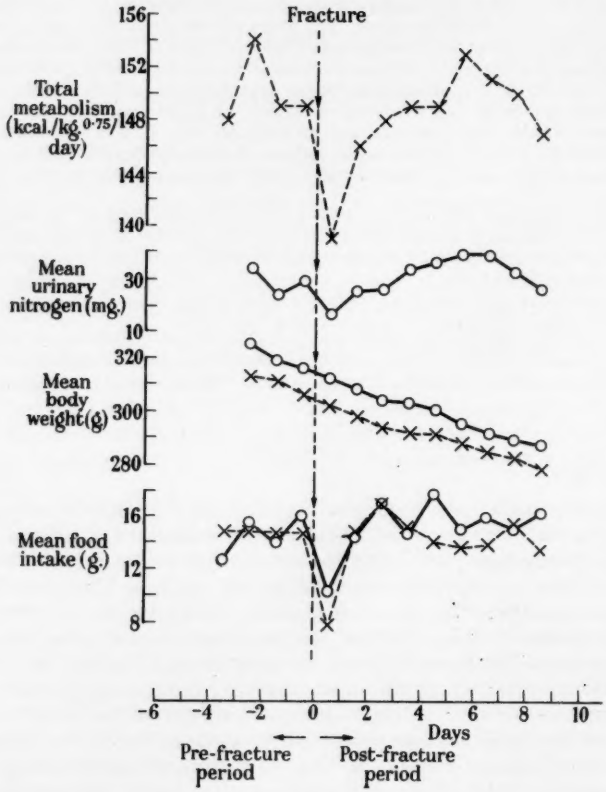

FIG. 1.—Total metabolism in kcal./kg.$^{0.75}$/day, mean weight, and mean food consumption of 9 rats on a high protein diet subjected to fracture of the left femur at day 0 (×———×). Also the protein metabolism in kcal./kg.$^{0.75}$/day of 6 rats on the same diet calculated from their urinary nitrogen excretion (○———○).

tion and on the third day reached a peak of 478 mg. N. Using the factors noted above, the heat provided by protein was calculated. Before fracture the level was 30 kcal./kg.$^{0.75}$/day, and the peak was 37 kcal./kg.$^{0.75}$/day.

The increase in protein metabolism following fracture was found to agree very closely in magnitude and shape with the increase in total metabolism. They are plotted together in Fig. 1.

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Vol. XXXVIII, No. 5, October 1957. (A. B. Cairnie, R. M. Campbell, J. D. Pullar and D. P. Cuthbertson.)

The figure entitled Figure 1, p. 506, should appear on p. 508 above the description given for Figure 2, and *vice versa*.

Re-examination of data on man

The older metabolic experiments on man (Cuthbertson, 1932) were re-examined to see whether the increased metabolism following injury could be as closely associated with the increased N excretion as in the foregoing experiments on the rat, or whether some part of the increase could be due to increased fat and carbohydrate utilisation. The only assumption made in the calculation is that 1 g. urinary N is associated with 5·9 litres oxygen (Brody, 1945). Using this factor the oxygen associated with protein oxidation can be calculated and, since the basal oxygen consumption is known, the oxygen associated with fat and carbohydrate can be found. In this way it can be seen whether or not the increased oxygen consumption is linked only to protein. In this re-analysis, taking first cases 5, 6 and 7, since in these pre-operation levels were available, it will be seen from Table I that the increase in the basal oxygen consumption is of the same order

TABLE I.—*Re-analysis of Published Data on Injured Men (Cuthbertson, 1932)*

Case. Nature of injury or operation	Days after injury	Basal oxygen consumption (ml./min.)	Total urinary nitrogen (g./day)	Oxygen used with protein (l./hr.)	Total oxygen (l./hr.)	"Non-protein" oxygen (l./hr.)
3.	−1	228	—	—	13·7	—
Fracture of tibia	1	236	14·28	3·5	14·2	10·6
	2	266	20·16	5·0	15·9	10·9
	3	284	23·31	5·8	17·1	11·3
	4	290	23·10	5·7	17·4	11·7
	5	297	25·51	6·3	17·8	11·5
	6	307	28·03	6·9	18·4	11·5
	7	299	26·04	6.4	18·0	11·5
	8	252	23·80	5·9	15·1	9·2
4.	2	250	11·06	2·7	15·0	12·3
Dislocation of	3	253	10·08	2.5	15·2	12.7
ankle	4	254	14.70	3.6	15.3	11·7
	5	254	16·66	4·1	15·3	11·2
	6	271	19·74	4·9	16·3	11·4
	7	291	18·48	4·6	17·5	12·9
	8	306	14·28	3·5	18·4	14·9
	9	308	12·88	3·2	18·5	15·3
	10	288	17·92	4·4	17·3	12·9
	11	269	17·72	4·4	16·2	11.8
5.	−2	217	12·18	3·0	13·0	10·0
Bone-grafting of	−1	221	11·86	2·9	13·3	10·4
an old un-united	1	214	No urine	—	12·8	—
fracture of humerus, using bone	2	198	23·41	5·8	11·9	6·1
	3	210	12·70	3·1	12·6	9·5
from tibia	4	220	17·53	4·3	13·2	8·9
6.	−1	260	6·82	1·7	15·6	13·9
Removal of torn	1	260	4·41	1·1	15·6	14·5
knee-joint cartilage	2	276	13·75	3·4	16·6	13·2
	3	303	16·06	4·0	18·2	14·2
	4	289	9·76	2·4	17·3	14·9
	5	265	9·45	2·3	15·9	13·6
	6	261	8·92	2·2	15·7	13·5
	7	258	8·61	2·1	15·5	13·4
7.	−1	253	9·14	2·3	15·2	12·9
Removal of torn	1	278	9·29	2·3	16·7	14·4
knee-joint cartilage	2	293	13·81	2·4	17·6	14·2
	3	276	13·18	3·3	16·6	13·3
	4	269	21·31	5·3	16·2	10·9
	5	271	21·00	5·2	16·3	11·1
	6	268	16·48	4·1	16·2	12·1

as the increase in oxygen requirement for the increase in protein metabolism calculated from the urinary nitrogen excretion. There can therefore be no definite increase in fat and carbohydrate metabolism for in these cases the protein metabolism is doubled, whereas fat and carbohydrate metabolism fluctuates at most 40 per cent.

In patients 3 and 4, which were accident cases and where no pre-trauma figures were in consequence available, it can be surmised that the increment in basal metabolism is also of the same order as the increase in oxygen requirement for the associated increase in protein metabolism.

Protein-free diet

In this experiment the rats were reduced to low endogenous urinary nitrogen excretion before commencing the metabolism studies. They were consequently in poor nutritive condition and lost weight steadily (Fig. 2) throughout the experi-

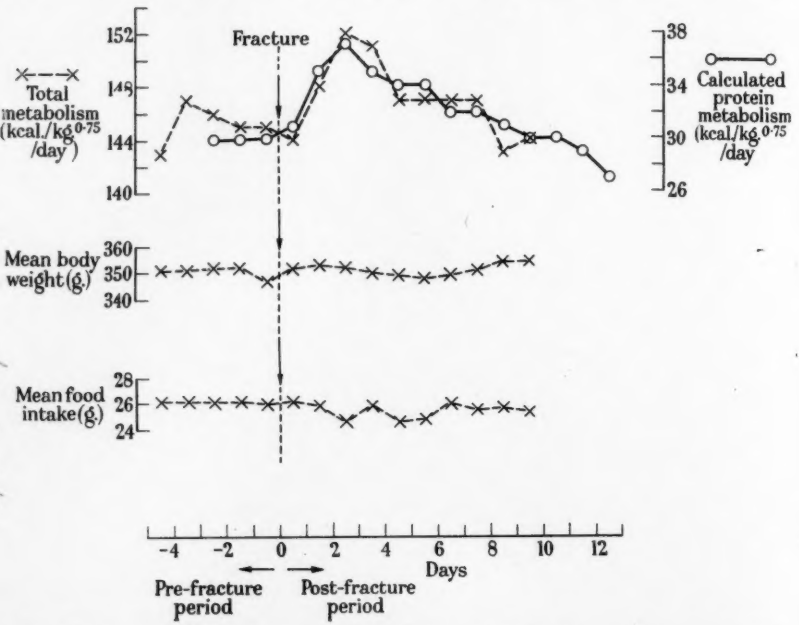

FIG. 2.—Total metabolism in kcal./kg. $^{0.75}$/day, mean weight, and mean food consumption of 9 rats on a protein free diet subjected to fracture at day 0 (×— — —×). Also the mean urinary nitrogen excretion, mean weight, and mean food consumption of 7 rats on the same diet (○———○).

ment, the average weight of the group of 9 rats used in the calorimeter falling from 313 g. to 278 g. in 11 days. Neither group ate well on the first day after fracture (Fig. 2). As reported previously by Munro and Cuthbertson (1943) the urinary N excretion does not show the characteristic and early increase (see Fig. 2); but there appears to be a small increase reaching a peak of 10 mg. N after

six days. Such an increase corresponds to an increase in metabolism of less than 1 kcal./kg.$^{0\cdot75}$/day. Measurement in the calorimeter showed that the total metabolism (Fig. 2) did not increase after fracture. On the first day it fell and recovered over the next two days to the pre-operation level. This fall can be explained in terms of reduced food intake.

DISCUSSION

The average total metabolism of the rats on a high protein diet remained at 145 kcal./kg.$^{0\cdot75}$/day (66·1 kcal./day) prior to injury and rose to a maximum of 152 kcal./kg.$^{0\cdot75}$/day (69·0 kcal./24 hr.) on the third day following injury which, according to previous investigations on rats (Cuthbertson *et al.*, 1939 ; Campbell *et al.*, 1954), is the time of the maximum excretion of nitrogen. The rise is primarily due to a rise in the minimum metabolic rate, which is increased by 7 per cent on the third day, and not in the maximum value reached during the day. This can be explained in terms of greater restlessness during the hours when the metabolic rate is normally low.

The body weight revealed little, if any, change in the course of the experiment with the high-protein diet but a decrease in tissue substance could have been masked by an increase in body water. It is known that there is an accumulation of fluid around the zone of injury and Wilkinson (1956) has drawn attention to the mobilisation of water from the tissues in the injured patient particularly during the period of oliguria which generally follows immediately after trauma.

The reported increase in the urea content of the urine after trauma shows that more protein is being deaminated. The heat obtained in the deamination process and in the use of the non-nitrogenous residue as a source of energy is found to account fully for the rise in metabolism found to occur. If the non-nitrogenous residues had been stored in some form or other less heat would have been produced. If a general acceleration of body metabolic processes had taken place, as on exposure to cold, fat would have been largely used as a source of increased heat. However, in this experiment it was found that protein metabolism alone was increased in response to trauma. It has been noted that urinary nitrogen does not rise following trauma in salt-maintained adrenalectomised rats, but the rise does occur in rats maintained on low levels of cortisone not themselves sufficient to provoke such a response (Campbell *et al.*, 1954). The question of the mode of action of trauma on protein metabolism remains to be answered ; the adrenal cortex is involved but is not the sole mediator.

It has been previously reported that rats fed a diet deficient in protein do not respond to trauma by increasing their urinary nitrogen. Our experiment shows that in circumstances where protein is not readily available, if indeed it is available at all in the quantities required, fracture does not lead to increased heat production. This is further evidence in support of the postulate that trauma increases protein metabolism only, and not fat and carbohydrate metabolism.

It may be that this elevation of metabolism—this traumatic fever—is a reaction of the body to enhance the healing process by accelerating the reactive processes which involve both inflammation and repair. Our re-examination of old data on man (Cuthbertson, 1932) has revealed that during the first week following injury the energy liberated by the oxidation of protein, when calculated as equivalent to the nitrogen excreted, can account for the rise in basal oxygen consumption

observed and reveals no corresponding increment in the oxidation of fat and carbohydrate.

These phenomena are the result of the reactions of the severely injured animal which in the natural wild state has to depend on responses which will continue to supply from the tissues of its own body, firstly, the continuing daily requirements of energy and water to maintain life and, secondly, the agents necessary to produce an inflammatory reaction and so to start the repair process. Such primitive reactions to injury also take place in the presence of an apparently adequate supply of food and it may be we are witnessing in such circumstances the superimposition of this primitive reaction on the normal metabolism of the adequately nourished animal, for in the wild state the severely injured, but otherwise well nourished animal at the time of the incident may be without access to food and water for several days. But if the readily metabolisable pool of body protein be exhausted by living beforehand on a low protein intake then both the increased metabolic rate and the increased oxidation of protein appear to be absent. Healing under such circumstances is delayed. Wilkinson (1956) has pointed out that these large disturbances of bodily composition assist the patient in recovering from a major operation. Then, as in the case of an abdominal operation, the consumption of food may be stopped or minimal for several days and much limited for several days, and the water intake may be reduced unless it is maintained by intravenous infusion.

SUMMARY

The average total metabolism of rats on a high protein diet as measured in a direct gradient layer calorimeter, was 145 kcal./kg.$^{0.75}$/day (66·1 kcal./day) prior to fracture of one femur, and rose to a maximum of 152 kcal./kg.$^{0.75}$/day (69·0 kcal. /24 hr.) on the third day following injury. This is the time of the maximum excretion of nitrogen (as urea) in the urine. The rise was primarily due to a rise in the minimum metabolic rate which was increased by 7 per cent on the third day.

The heat calculated as derived from the oxidation of protein corresponding to the extra urinary nitrogen excreted, agreed very closely in magnitude and shape with the increase in total metabolism. There was no definite increase in fat and carbohydrate metabolism.

A re-examination of data derived from studies of injury in man revealed that during the first week following the trauma the calculated increase in oxygen consumption corresponding to the rise in urinary nitrogen, was of the same order as the increment in the basal consumption of oxygen.

Rats maintained on a practically protein-free diet did not exhibit the characteristic sharp rise in urinary nitrogen excretion following fracture—presumably because the labile protein reserve pool was exhausted—and there was no rise in the total metabolism as measured in the calorimeter.

The severely injured animal in the natural wild state has to depend on responses which will continue to supply from the tissues of its own body, first, the continuing daily requirements of energy and water to maintain life and, second, the agents necessary for repair. Such primitive reactions to injury also take place in the presence of an apparently adequate supply of food. But if the readily metabolisable pool of body protein be exhausted beforehand on a low protein intake then both the increased metabolic rate and the increased oxidation of protein are absent.

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THE EFFECT OF ASPIRIN, CORTISONE AND OTHER COMPOUNDS ON SUSCEPTIBILITY TO ANAPHYLACTIC SHOCK IN MICE

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Of the anti-rheumatic compounds acetyl salicylic acid (aspirin), cortisone and γ-resorcylic acid, aspirin was found to protect rabbits from anaphylactic shock (Campbell, 1948; Lepper, Caldwell, Smith and Miller, 1950) and cortisone (Nelson, Fox and Freeman, 1950) to protect mice. In the present experiments these three and two other compounds, 2 : 4-dinitrophenol and tri-iodothyronine, were examined for anti-allergic activity in the mouse.

γ-Resorcylic acid, structurally related to salicylic acid, was shown by Reid, Watson, Cochran and Sproull (1951) to be as effective as salicylate in the treatment of rheumatic fever. It has the ability to form a double chelate ring and was chosen by these authors for investigation to test the hypothesis that the therapeutic action of salicylate lies in its ability to form a chelate ring. The metabolic stimulants 2 : 4-dinitrophenol and tri-iodothyronine were examined for anti-allergic activity following the finding that salicylate in addition to being anti-rheumatic acts as a metabolic stimulant in man (Cochran, 1952) and in rabbits (Reid, 1957).

MATERIALS AND METHODS

Mice.—W-Swiss mice of both sexes, weighing 30–40 g. were used in all experiments: animals of this weight were chosen because of the impression formed that it was more difficult to elicit anaphylactic shock in smaller animals. The mice were bred in the Department from stock obtained from the Agricultural Research Council's Field Station at Compton.

Bovine fibrinogen and bovine γ-globulin—Armour Biochemicals.

Acetyl salicylic acid—B.D.H.

2 : 4-*Dinitrophenol*—Hopkin and Williams.

Propylene glycol—B.D.H.

Sodium γ-resorcylate—Synthesized in the Department.

Cortisone—Cortisone acetate B.P.(" Cortistab ", Boots Pure Drug Co. Ltd.).

Tri-iodothyronine—Synthesized in the Department.

Sensitization and challenge.—All mice were sensitized and challenged as described in a previous report (Cameron, 1956): 3 sensitizing injections of antigen were given intraperitoneally at intervals of 7 days and the challenging injection intravenously 7 days after the last sensitizing injection; 30 mg. (3 doses of 10 mg.) of bovine gamma globulin and a challenging dose of 1 mg. were given; the corresponding doses for bovine fibrinogen were 1·0 and 0·35 mg. respectively. As much as 10 mg. of the antigens injected intravenously was without visible effect in unsensitized control mice.

Assessment of shock.—Following challenge the mice appear normal—if anything hyperactive—for about 5 min., then start to scratch the nose and ears vigorously; the face begins to swell and with the ears becomes flushed, the eyes watery and red-rimmed. In the next 5–10 min. there is prostration, the animals moving only when disturbed; breathing is slow and deep and there is a marked fall in body temperature. They lie thus for a further 10 to 15 min., the tail and pads becoming cyanotic, and about 15 min. later begin to recover; after

30 min. they may be sitting, still hunched up, fur bristling, slowly returning to normal. In fatal shock death usually occurs within 1 hr. of challenge, prostration being followed by gasping breathing, sometimes convulsions at irregular intervals, and finally death.

Pre-treatment of mice

Aspirin.—Mice were pre-treated with aspirin for 3 days, beginning on the fourth day after the last sensitizing injection of antigen, following the suggestion of Derrick, Hitchcock and Swift (1928) that salicylates affect antigen-antibody reactions by depressing the level of circulating antibody. The maximum tolerated subcutaneous dose of aspirin dissolved in propylene glycol was found to be about 0·75 mg. in 0·005 ml. per g. mouse weight (with a maximum of 25 mg. per mouse) and the blood level—80–100 mg. per cent, estimated as sodium salicylate—to be maximal 45–75 min. after injection, with some aspirin still detectable 12 hr. later. Animals were given 7 subcutaneous injections of 0·75 mg. per g. in about 0·15 ml. of propylene glycol at intervals of 12 hr. and were challenged 45–75 min. after the last injection, *i.e.*, when the aspirin blood level was maximal.

Cortisone.—Two subcutaneous 0·1 ml. injections, each of 1 mg., were given 24 hr. apart on the fifth and sixth days after the last sensitizing injection, and the mice were challenged 18 hr. after the second injection. This dosage was based on that of Nelson *et al.* (1950) who found that 3 mg. intramuscularly protected mice from fatal anaphylactic shock.

2 : 4-*Dinitrophenol.*—The maximum tolerated subcutaneous dose of the sodium salt of 2 : 4-dinitrophenol was found to be about 0·02 mg. per g. body wt. The blood level was found to be maximal—7 to 9 mg. per cent—during the first hour after injection and none could be detected 6 hr. later. Accordingly, mice were pre-treated for 3 days, beginning, as with aspirin, on the fourth day after the last sensitizing injection, and were given 7 subcutaneous injections of about 0·5 ml. of 0·02 mg. per g. of an aqueous solution of 2 : 4-dinitrophenol at intervals of 12 hr. They were challenged 30 min. after the last injection, *i.e.*, when the dinitrophenol blood-level was maximal.

Sodium γ-resorcylate.—It was reported by Buttle (1951) that mice can tolerate 1 mg. per g. of the sodium salt of γ-resorcylic acid and that excretion, as found in the rat, is rapid, being almost complete within 4 hr. Hence mice were given 3 subcutaneous injections of about 0·5 ml. of 1 mg. per g. sodium γ-resorcylate at intervals of 6 hr. on the sixth day after the last sensitizing injection and were challenged 30 min. after the last injection.

Tri-iodothyronine.—Mice were given subcutaneously 2 arbitrary doses of 15 μg. at intervals of 24 hr., the first injection on the fifth day after the last sensitizing injection. They were challenged 18 hr. after the second injection.

RESULTS

In confirmation of the results of Nelson *et al.* (1950) cortisone was found to afford complete protection against anaphylactic shock in mice sensitized to bovine

TABLE—*Protective Effect of Five Compounds against Anaphylactic Shock in Mice.*

Compound	Mice sensitized to bovine fibrinogen. Number of mice: Challenged	Shocked*	Unaffected	X^2 (shocked/unaffected, N = 1)
None (controls)	45	40 (27)	5	—
Cortisone	47	1 (1)	46	67
Aspirin	55	44 (25)	11	1·5
2 : 4-Dinitrophenol	49	40 (28)	9	1·0
2 : 4-Sodium γ-resorcylate	44	41 (16)	3	0·5
Tri-iodothyronine	45	40 (25)	5	0
	Mice sensitized to bovine γ-globulin.			
None (controls)	33	27 (7)	6	—
Cortisone	54	0	54	64·1
Aspirin	42	25 (7)	17	4·3
2 : 4-Dinitrophenol	51	40 (10)	11	0·14
2 : 4-Sodium γ-resorcylate	46	40 (8)	6	0·4
Tri-iodothyronine	45	38 (8)	7	0·1

* Figures in brackets denote number of mice fatally shocked.

γ-globulin and to bovine fibrinogen. Aspirin on the other hand afforded no protection to mice sensitized to bovine fibrinogen and possibly some protection to mice sensitized to bovine γ-globulin ($N = 1$, $X^2 = 4{\cdot}3$, $P = 0{\cdot}05$). It is interesting that, in the aspirin-treated groups, shock where it occurred was just as severe as in the untreated control groups although the aspirin-treated animals recovered in about half the time.

None of the other compounds showed any protective action. On the contrary, tri-iodothyronine appeared to hasten the onset of shock and γ-resorcylic acid to prolong it.

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SOME EFFECTS OF PHENOLIC ANTI-OXIDANTS ON SODIUM AND POTASSIUM BALANCE IN THE RABBIT

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THIS paper is part of a study of the biological activity of two commercial anti-oxidants, 2 : 6-di-*tert.*-butyl-4-methylphenol (BHT) and butylated hydroxyanisole (BHA), which is a mixture of two isomers, 85 per cent being 2-*tert.*-butyl-4-methoxyphenol and 15 per cent 3-*tert.*-butyl-4-methoxyphenol. These anti-oxidants are used for the stabilization of edible fats. In the course of metabolic studies of BHA (Dacre, Denz and Kennedy, 1956) and BHT (in progress), it was found that a series of daily doses of 1 g. to rabbits led to muscular weakness, inanition and death. Although it is well known that large doses of such phenolic materials are lethal, the mechanism of poisoning has been obscure. It will be shown here that large doses of BHA and BHT produce a gross disturbance of sodium, potassium and water balance in the rabbit.

METHODS

Animals

The rabbits were does of the New Zealand white strain weighing 1·3–2·0 kg. They were maintained on a standard pellet diet (wheat, 40 per cent ; pollard, 33 per cent ; bran, 27 per cent), and were kept singly in metabolism cages designed to permit the separate collection of urine and faeces. Except where otherwise indicated in the text, water and food were supplied *ad lib.* The daily intake of food and water and the urine volumes were measured. The faeces were generally dry and well-formed and were easily collected and weighed.

Materials and dosage

BHA (Sústane) was obtained from the Universal Oil Products Company, Riverside, Illinois, U.S.A., and BHT from the Shell Company of New Zealand, Ltd. Both BHA and BHT were dissolved in olive oil and 1 g. in 5 ml. of oil was administered by stomach tube to the rabbits each morning. Rabbits usually refused food during the 24 hr. after receiving BHA or BHT. Rabbits given 5 ml. of olive oil also showed a marked reduction in food intake.

Histology

Frozen sections of the adrenal were cut at 10 μ and stained with Oil Red O. Paraffin sections of tissues were stained with Harris's haematoxylin and eosin, Crossman's trichrome and Heidenhain's azan.

Sodium and potassium estimations

All estimations of Na and K were made by flame photometry.

In urine.—Direct determinations were made on appropriate dilutions of urine.

In serum.—Blood was collected from the marginal vein of the ear into silicone-coated tubes. The serum was separated by centrifuging the blood immediately after collection (cf. Cardus and Llaurado, 1955).

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In food and faeces.—About 1 g. of the material was weighed into a micro-Kjeldahl flask and digested with redistilled HNO_3 until the residue was colourless. This residue was taken up in 10 ml. of 0·1 N-HCl and the Na and K determined directly.

In tissues.—Rabbits were killed in a gas chamber and the muscles (tibialis anticus, soleus, biceps femoris), the diaphragm, heart and brain were dissected and samples with a wet weight of 0·3–1·0 g. were taken into weighed stoppered tubes. After reweighing, the tubes were placed in a deep-freeze cabinet for 2–4 hr. The tissues were dried from the frozen state. They were then weighed to give dry weight and the fat was extracted by shaking with 10 ml. of dry ether (dried by distillation from conc. H_2SO_4). A constant weight for extracted tissue was obtained for the biceps femoris, soleus, tibialis anticus and heart after one extraction, diaphragm after two and brain after three consecutive extractions with dry ether. The dry tissue was dissolved in 5 ml. concentrated HNO_3 (redistilled) and transferred to a micro-Kjeldahl flask and digested until the residue was white. The residue was taken up in 10 ml. of 0·1N-HCl for Na and K determinations.

Aldosterone assays

Urine produced by 3–4 rabbits in 24 hr. was pooled and 40 min. after acidification to pH 1 extracted 4 times with chloroform and then re-extracted 4 more times after 24 hr. The extract was evaporated to dryness at reduced pressure, taken up in a small volume of chloroform, dried in a stream of nitrogen, dissolved in 20 per cent ethanol in water and the concentrate assayed on adrenalectomized rats. The rats used in the bio-assay were injected with a portion of the extract and a load of Na and K. The activity of aldosterone was measured by the reduction of the Na/K ratio in the urine of the adrenalectomized rats and expressed as a percentage of the Na/K of a control group injected with solvent alone and given the same load of Na and K on the same day. The figures obtained as percentages have been converted to μg. of aldosterone by a calibration curve obtained previously with pure aldosterone. This method has been fully described by Llaurado (1956*a*, *b*).

Extracellular fluid volume

The thiocyanate method was used. The rabbit's bladder was emptied by suprapubic pressure. A 2 ml. sample of blood was taken from the left ear into a siliconed tube. A dose of 25 mg. of sodium thiocyanate in 2·5 ml. of water was injected slowly into the marginal vein of the right ear. Two hours later the rabbit's bladder was again emptied and a second sample of 2 ml. of blood taken from the left ear. Protein-free filtrates were obtained from the separated serum with 10 per cent trichloracetic acid. The thiocyanate in the protein-free filtrates of serum and in the urine were determined by measuring in a spectrophotometer the intensity of colour developed with ferric nitrate (see Elkinton and Taffel, 1942).

The extracellular fluid volumes are expressed as ml. Rabbits given BHA or BHT lose weight and this loss obscures the fall in extracellular fluid volume that occurs during treatment when the results are expressed as a percentage of body weight. Rabbits with similar initial weight (1·30–1·35 kg.) were chosen for this experiment. The mean value of the extracellular fluid volume of rabbits before dosing with BHA was 416 ml. or 31·5 per cent of body weight.

RESULTS

Increase in urinary sodium and potassium

When daily doses of 1 g. of BHA or BHT are given orally to rabbits there is a ten-fold increase in sodium excretion in the urine. At the same time there is a

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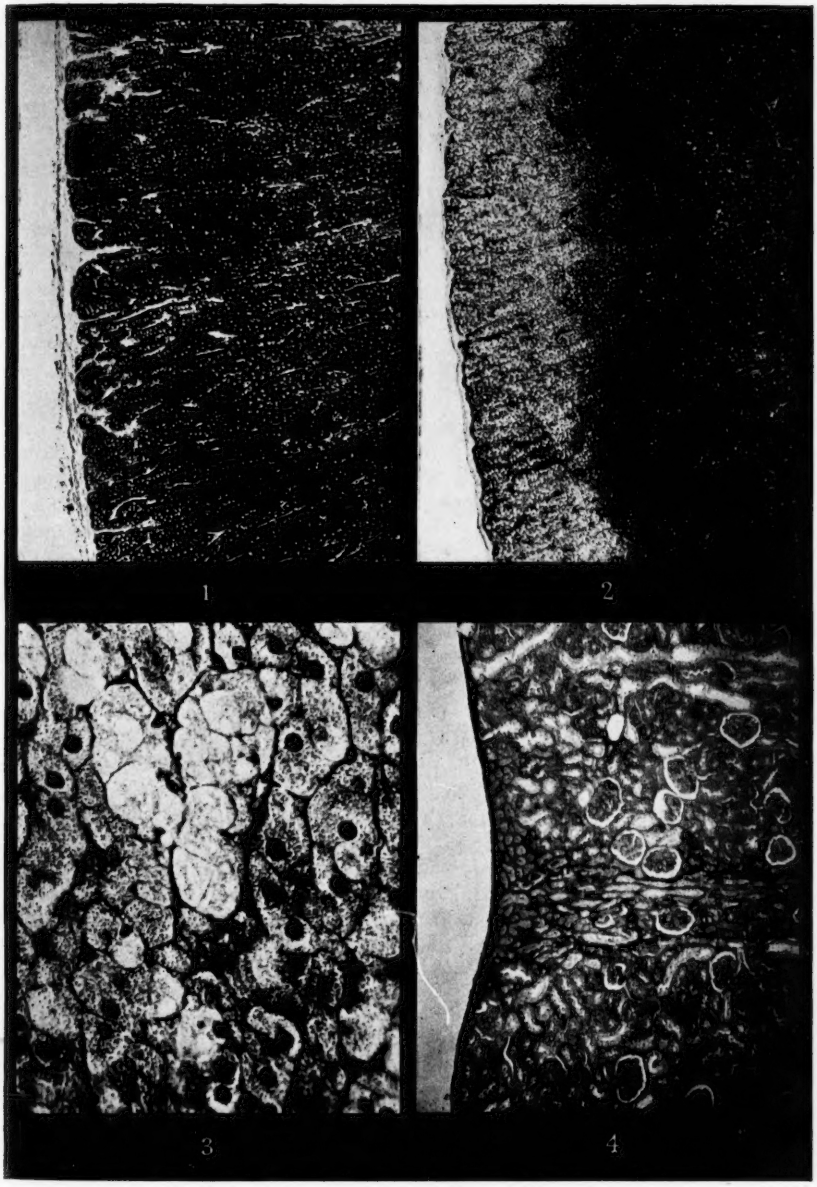
EXPLANATION OF PLATES

FIG. 1.—Z. glomerulosa and fasciculata of normal rabbit adrenal. Stained with Oil Red O.

FIG. 2.—Rabbit adrenal after five doses of BHA showing lipoid depletion in Z. glomerulosa. Stained with Oil Red O.

FIG. 3.—Rabbit adrenal after five doses of BHA. Inner fasciculata showing cells undergoing hydropic degeneration.

FIG. 4.—Kidney from control rabbit showing scarring and fibrosis.



Denz and Llaurado.

20 per cent increase in potassium output in the urine. The results obtained with BHA are given in Table I. Similar increases have been obtained with BHT.

TABLE I.—*Effect of Daily Dose of One Gram of BHA on Urinary Sodium and Potassium (as m-equiv./Day)*

	Basal level	Number of doses				
		1	2	3	4	5
Sodium	0·137	0·787	1·179	1·300	0·815	1·063
Potassium . . .	5·394	6·690	5·847	6·058	5·130	5·260

Results are means for 13 animals.

Changes in the adrenal cortex

The normal appearance of the adrenal cortex of the rabbit when stained with Oil Red O is shown in Fig. 1. After a series of three to five doses of BHA or BHT there is a gross depletion of lipoid staining of the zona glomerulosa (Fig. 2). The intensity of staining of the other zones of the adrenal cortex is unaffected. Sections stained with haematoxylin and eosin do not show any cellular necrosis in the zona glomerulosa, but show some increase in cytoplasmic basophilia (cf. Symington and Davidson, 1956).

In sections stained with haematoxylin or by trichrome methods, single cells and small groups of cells in the zona fasciculata and especially in its deeper layers (the inner fasciculata of Nicander, 1952) are seen to be undergoing hydropic degeneration (Fig. 3). In some sections from control animals similar changes are seen occasionally in the zona reticularis but not in the zona fasciculata. In this paper no attempt will be made to evaluate the significance of the changes in the zona fasciculata.

Changes in aldosterone excretion

The remarkable increase in sodium excretion and the loss of lipoid staining in the zona glomerulosa, which seems to be the most likely site of the production of aldosterone (Deane, Shaw and Greep, 1948 ; Peschel and Race, 1954, and others), showed the need for data on aldosterone excretion in these rabbits.

A biological assay was used. For convenience of exposition the assays are expressed as μg. of aldosterone per day although no great weight is attached to the absolute figures owing to the difficulties that have been found in comparing results obtained in different laboratories (see Dodds, Garrod and Simpson, 1956). The changes in aldosterone level occurring with treatment are regarded as being more significant than the individual figures for aldosterone.

In rabbits treated with BHA or BHT there is a consistent rise in aldosterone excretion (Fig. 5). The average level of aldosterone excretion in normal rabbits was 0·027 μg. per day with a range of 0·003 to 0·061 μg. After dosing with BHA or BHT a high level of aldosterone excretion was maintained for periods up to one week. One group of four rabbits excreted an average per rabbit of 0·044 μg. on the seventh day of treatment compared with an initial level of 0·017 μg. The highest aldosterone levels were reached about the fourth day of treatment (levels of 0·15–0·19 μg.). The later samples showed some decrease in aldosterone but did not fall to pre-injection levels.

Renal changes

The association of a raised excretion of both sodium and aldosterone suggests that the increased sodium loss is due to changes in renal function. An examination of the kidneys of rabbits from our colony shows that all kidneys are affected by a chronic inflammatory process normally described as pyelonephritis (Fig. 4). The focal lesions of the kidney appear in the rabbits in the first three or four weeks of life before they are weaned. These lesions are sufficiently severe to prevent

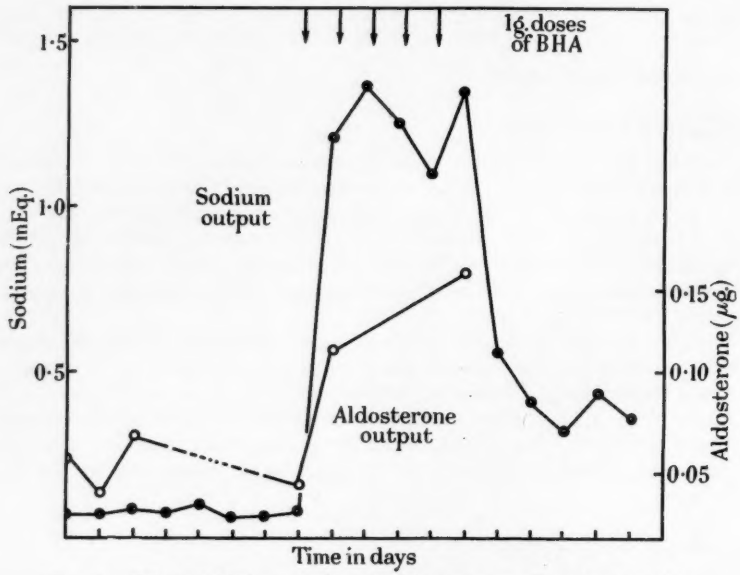


FIG. 5.—Effect of BHA on the Na and aldosterone excretion in the rabbit.

any detailed histochemical studies of the kidneys. In these acute experiments there was no evidence of vacuolation of the tubular epithelium which has been described in chronic potassium deficiency in man and some experimental animals (Conn and Johnson, 1956; Fourman, McCance and Parker, 1956, etc.).

Changes in serum sodium and potassium

The first three doses of BHA or BHT cause a slight rise in serum sodium. Only in the terminal stages is there any fall in the sodium level. Potassium, on the other hand, shows an immediate fall which is progressive and the level falls to about half the normal value after the sixth dose (Table II).

Sodium and potassium balance

The extent of potassium loss in treated animals determined by balance experiments is greater than might be expected from data on urine alone. Figures in Table I show that an increase of 2·01 m-equiv. of potassium above normal levels

TABLE II.—*Effect of Daily Doses of One Gram BHA on m. equiv./l-Sodium and Potassium in the Serum of Rabbits*

	Basal level	Number of doses 1	2	3	4	5	6	7
Sodium	146	148	150	150	145	145	142	126
Potassium	4·0	3·7	3·2	3·2	2·7	2·7	2·2	1·9

Each figure is the mean of four estimations.

was excreted in six days. This observation fails to take into account the fact that animals dosed with the anti-oxidants in olive oil ceased to eat. This reduces their potassium intake to zero and they are then in negative balance to the extent of the potassium in the urine and faeces. The effect on the potassium balance is shown in Fig. 6, where in the course of a series of six doses of BHA the rabbit

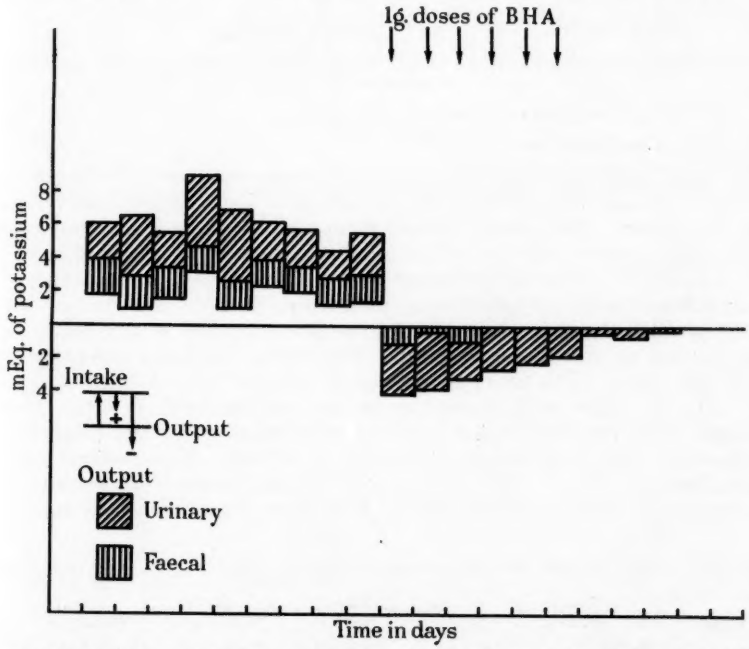


FIG. 6.—Effect of six daily doses of 1 g. of BHA on the K balance in the rabbit. Total K loss 36 m-equiv.

has lost 36 m-equiv. of potassium, which was about half of the potassium normally present in the body. Olive oil alone does not produce any significant potassium loss because the potassium output falls sharply with the decrease in food intake. The effect on sodium balance is less remarkable, and the corresponding sodium loss after 6 doses is 5·7 m-equiv.

The relative proportions of the daily losses of sodium and potassium to the amounts in the extracellular fluids and the cells is shown in Fig. 7. It can be

seen that the daily loss of sodium is unlikely to produce any marked effect on the sodium in the extracellular fluid, and therefore in the serum, for some days. The daily loss of potassium is grossly in excess of the amount in the extracellular fluid at any one time and can be maintained only by a continual withdrawal of potassium from the cells.

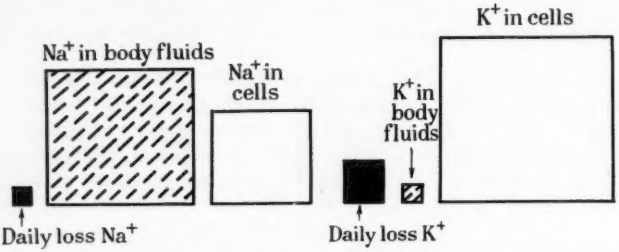


FIG. 7.—Comparison of daily loss of Na and K in treated rabbits with the total intracellular and extracellular Na and K.

Tissue sodium and potassium levels

The data for sodium and potassium are those obtained by direct analysis and no attempt has been made to divide them into extra- and intracellular levels within the tissue. The tissues chosen were white muscle (biceps femoris), red muscle (soleus), mixed red and white muscle (tibialis anticus), diaphragm, heart muscle and brain. These tissues show very different levels (Table III), and they respond differently to the loss of body potassium which occurs when either BHA or BHT is administered. In voluntary muscle, there is a decrease in muscle potassium and an increase in sodium. This change becomes accentuated after four or five days. The changes are shown for the tibialis anticus muscle in Fig. 8. On the other hand, the potassium and sodium levels of brain, heart and diaphragm show very little effect until the terminal stages when, about the sixth day, there is a fall in potassium and a rise in sodium. These effects are shown for heart muscle in Fig. 9. From these results it can be seen that the intracellular potassium is not drawn on uniformly at a time when potassium loss is considerable.

TABLE III.—*Sodium and Potassium in μ. equiv./g. Wet Tissue in Normal Rabbits*

Tissue	Biceps femoris	Soleus	Tibialis anticus	Diaphragm	Heart	Brain
Sodium	15·1±0·7	29·7±1·6	19·6±0·6	25·6±1·3	46·0±3·0	46·8±2·2
Potassium	88·0±4·5	82·2±4·1	94·3±1·2	63·2±2·5	62·9±1·2	71·4±2·5

Means and S.E.M. of 5 estimations.

Cause of death

The profound fall of serum potassium and the reduction in cellular potassium especially in the later stages of poisoning suggests that the rabbits are dying because of potassium deficiency. This conclusion is supported by the observation that the rabbits develop muscular weakness especially in the hind limbs. Rabbits can be kept alive by administering 1 per cent potassium chloride. Some of the

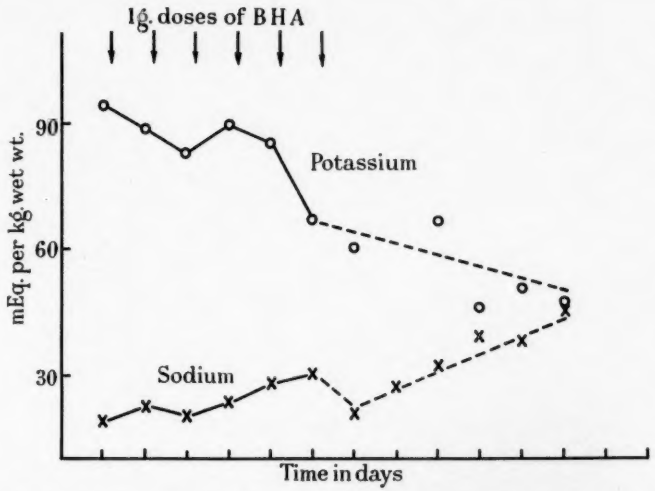


FIG. 8.—Effect of BHA on the Na and K levels in the tibialis anticus muscle. First six points on each curve are means of five animals, remaining points from single animals.

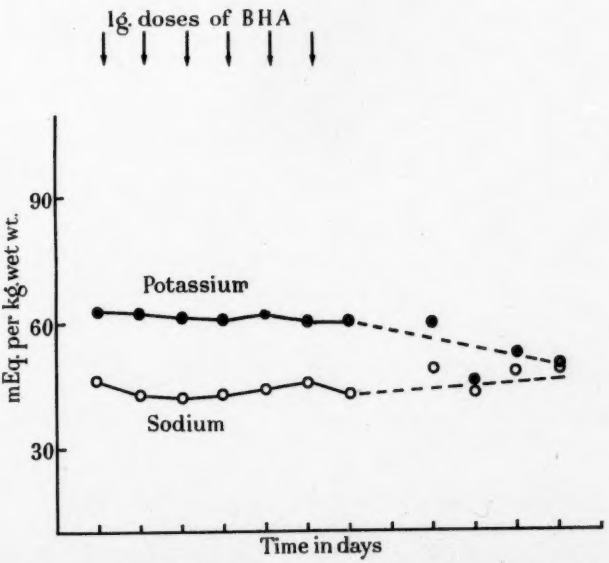


FIG. 9.—Effect of BHA on the Na and K levels in the heart muscle. First six points on each curve are means of five animals, remaining points from single animals.

affected animals will drink this solution. Others had to receive it by stomach tube.

These findings explain an earlier observation that rabbits which were fed cabbage survived many more doses of BHA or BHT than those supplied only with food cubes. It also explains the failure of sodium chloride therapy which was attempted when the sodium-losing effect was first detected. Replacement therapy with 9-α-fluorohydrocortisone acetate in an attempt to prevent the sodium loss was also tried before the aldosterone assays had been made. In view of the fact that aldosterone excretion was at a high level all the time and that death was due to potassium loss, it was not surprising that corticoid replacement therapy failed to keep the rabbits alive.

Extracellular fluid volume

It has been shown above that in treated animals there is an increased loss of sodium in the urine and an increase in the sodium content of muscle. This happens at a time when the sodium intake has been reduced almost to zero so

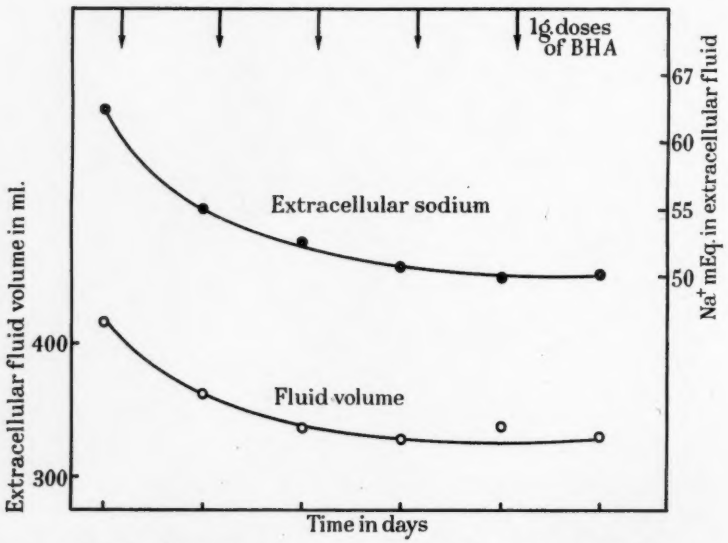


FIG. 10.—Effect of BHA on the extracellular fluid volume and the total extracellular sodium. Each point is a mean of four estimations.

that this movement can only happen at the expense of the sodium in the extracellular fluid. It has been shown that the concentration of sodium in the serum, and hence in the extracellular fluid, does not fall at least for the first five days. These observations are compatible only with a fall in extracellular fluid volume, and this has been found. In Fig. 10 the total amount of extracellular sodium is shown and it will be seen that there is an early fall in the amount of extracellular sodium that continues over the period of five days during which the animals are dosed with the anti-oxidant.

DISCUSSION

The death of rabbits given 1 g. daily of BHA or BHT is attributed to the loss in the urine of potassium which leads to a fall in the potassium in the muscle and other tissues.

The association of high aldosterone output with excessive potassium loss in man was first reported by Cope and Llaurado (1954), although the significance of this association was not properly understood until Conn (1955) showed that it is due to a hyperfunctional adrenal tumour, and established the clinical syndrome called primary aldosteronism. Subsequently it has been shown (Luetscher and Curtis 1955; Falbriard, Muller, Crabbé and Duckert-Maulbetsch, 1955) that a high aldosterone output can be also associated with excessive sodium loss in the so-called "sodium-losing" nephritis. In this case the primary lesion consists of an inability of the renal tubules to reabsorb sodium, and the hyperproduction of aldosterone is a compensatory mechanism to save body sodium. In the long run, however, this mechanism is detrimental because it may lead to potassium depletion.

In the experiments reported here the association of an increased excretion of sodium in the urine with an increased output of aldosterone suggests that the primary effect of the anti-oxidants is renal and the increased aldosterone secretion is secondary to the sodium loss. The histological picture of selective depletion of lipoid of the zona glomerulosa of the adrenal supports the current view that this is the site of aldosterone production. The relatively high level of aldosterone secretion even in the later stages of poisoning suggests that the loss of lipoid is not evidence of failure of the adrenal, but is a measure of the rapid production of aldosterone.

The underlying cause of the excessive loss of electrolytes in the urine has not been found. Both BHA and BHT are excreted mainly as glucuronides and ethereal sulphates. These materials are comparatively strongly acid and they may carry sodium and potassium with them into the urine. However there is very little increase in ammonium ion in the urine. There is the further possibility that BHA and BHT by their action as anti-oxidants may have a direct effect on the tubular epithelium of the kidney. Preliminary studies of the metabolism of BHT in the rabbit complementary to those already reported on BHA suggest that the metabolism of the compounds is very different. A striking finding in this paper has been the identical effects of these two compounds on the sodium, potassium and water balance in the rabbit.

SUMMARY

Two phenolic anti-oxidants when given to rabbits by stomach tube in doses of 1 g. per day produce similar effects on Na and K excretion in the urine: a ten-fold increase in Na and a 20 per cent increase in K.

The increase in urine Na has little effect on serum Na, and there is an early fall in extracellular fluid volume which parallels a similar fall in the total amount of extracellular Na.

The serum K falls steadily after five days of treatment, and this is associated with a fall in K and a rise of Na in voluntary muscle. Heart muscle shows little change in Na and K levels until the terminal stages.

The adrenal cortex shows a gross decrease in fat staining of the zona glomerulosa, the region believed to be the site of aldosterone production. At the same time there is an increased excretion of aldosterone in the urine.

A series of daily doses of either anti-oxidant leads to muscular weakness, inanition and death, which is attributed to the loss in the urine of K leading to a fall in the K in the muscle and other tissues.

It is suggested that the primary effect of the phenolic anti-oxidants is renal and that the increased aldosterone secretion is secondary to the Na loss. The underlying cause of the excessive loss of electrolytes in the urine has not been found.

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THE SYNTHESIS OF PROTEIN IN THE RAT CONNECTIVE TISSUE CELL

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ALTHOUGH the incorporation of some amino acids into the proteins of animal tissues has been extensively investigated, both in the living animal and in tissue slices, there has been no systematic study of the pattern of amino acid incorporation in a homogeneous population of living animal cells. Such a study, carried out on the connective tissue cell of the rat, is described in the present paper. Connective tissue cells can be isolated from the heart of the rat as a homogeneous suspension of single cells which can be cultivated *in vitro* under conditions where the rate of multiplication can be accurately measured (Harris, 1955*a*). The growth characteristics of animal cells cultivated in this way are in many respects similar to those of a bacterial culture, and the results obtained with such preparations may be compared with the results of similar investigations carried out on bacteria (Roberts, Abelson, Cowie, Bolton and Britten, 1955). In the present study the connective tissue cells were freshly isolated from the animal for each experiment; the pattern of amino acid incorporation described may therefore serve as a basis for comparisons with other normal tissue cells and with malignant cells cultivated under similar conditions.

EXPERIMENTAL

The connective tissue cells were obtained from the hearts of 5-day-old rats. The technique for preparing a homogeneous population of single cells and cultivating them in coverslip chambers has already been described (Harris, 1955*a*). The cells were freshly isolated from the heart tissue for each experiment and were grown in a medium consisting of 50 per cent of bovine serum and 50 per cent of Hanks' solution (Hanks, 1948). Since it had been shown that the survival of these cells *in vitro* required the presence of certain sulphur-containing compounds (Harris, 1955*b*), L-cysteine hydrochloride was added to the medium at a concentration of 0·001 M.

With the exception of histidine, tryptophan, methionine and cystine, all the radioactive amino acids were biologically prepared L-isomers uniformly labelled with ^{14}C. Uniformly labelled histidine, tryptophan, methionine and cystine were not available; L-histidine (2-ring-^{14}C), DL-tryptophan (indolyl alanine-3-^{14}C), DL-methionine-^{35}S and DL-cystine-^{35}S were used. The uniformly labelled glucose-^{14}C was biologically prepared.

Each labelled amino acid was added to the medium to give a final concentration of 2–4 μc. per ml. This represented an addition of between 10^{-3} and 10^{-4} millimols of amino acid per ml. of medium. When labelled glucose was used, glucose was omitted from the Hanks' solution in order to reduce the amount of carrier present. The labelled glucose was added to the medium at a concentration of 7 μc. per ml.

About 12 culture chambers containing a total of 50,000–100,000 cells were used for each experiment. The cells were grown in the medium containing the labelled compound for 24 hr. The medium was then withdrawn from the chambers, and the cells, still adherent to the glass coverslips, were washed with two changes of physiological saline. The cells

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were disrupted and completely removed from the coverslips by filling the chambers with distilled water containing about 1 per cent of the detergent "Teepol." The fluid containing the disrupted cells was withdrawn from the chambers and an equal volume of 30 per cent (w/v) trichloracetic acid was added to it. The precipitate was spun down and washed three times with distilled water (3 ml.). The supernatant and the washings were discarded. Nucleic acids were extracted from the precipitate with 5 per cent trichloracetic acid (3 ml.) at 90° for 30 min., and lipids with a mixture (3 ml.) of equal parts of ether and 75 per cent ethanol at 40°–50° for 30 min. The residue was hydrolysed with 6N hydrochloric acid (0·5 ml.) at 105° in sealed pyrex tubes. Hydrolysis was usually continued for 18 hr., but shorter periods were also used when labelled tyrosine, histidine and methionine were being studied, since these compounds underwent some degradation during hydrolysis. When labelled tryptophan was used an alkaline hydrolysis was carried out: the residue was heated in a sealed tube for 18 hr. with a saturated solution of barium hydroxide. Carbon dioxide was subsequently passed through the hydrolysate to remove most of the barium hydroxide.

The hydrolysate was dried down, taken up in 5–10 µl. of water and subjected to two-dimensional chromatography on paper. The solvents used were butanol-acetic acid-water (76 : 18 : 76 by vol.) in the first dimension and phenol-water (500 g. in 120 ml.) in the second. These solvent systems gave a satisfactory resolution of most of the amino acids in the protein, but valine was not resolved from methionine, nor leucine from isoleucine, and the basic amino acids, histidine, arginine and lysine, were poorly separated. Methionine was resolved from valine by oxidizing the hydrolysate with hydrogen peroxide (100 vol. per cent) for 15 min. at 105°, the methionine being converted to methionine sulphone. The basic amino acids were resolved by a combination of ionophoresis and chromatography. The hydrolysate was subjected to ionophoresis on paper for 2 hr. at 500 v in a volatile buffer of collidine acetate at pH 7 (Newton and Abraham, 1954), and then to chromatography in butanol-acetic acid-water. No attempt was made to resolve leucine from isoleucine.

Autoradiographs on X-ray plates were made from the chromatograms. All the radioactive areas shown on the X-ray film were traced out on the corresponding chromatogram and then counted directly with a suitably mounted thin end-window Geiger-Müller tube. The chromatogram was then sprayed with ninhydrin and the radioactive areas corresponding to the various amino acids were identified. The radioactivity in each amino acid was expressed as a percentage of the total radioactivity in all the amino acids present, that is, in the whole hydrolysate. Tyrosine, histidine, tryptophan, methionine and cystine, which underwent some degradation during hydrolysis or on the paper during chromatography, presented special problems. Radioactive degradation products from these amino acids appeared on the autoradiographs. Except for tryptophan, none of these degradation products appeared in the same position as any other amino acid. A small amount of labelled glycine was produced by degradation from the labelled tryptophan. In the case of a labelled amino acid which neither received carbon from nor contributed carbon to any other amino acid, degradation during hydrolysis or chromatography gave rise to no difficulty in the interpretation of the results: all the radioactivity in the hydrolysate was found in that amino acid and its degradation products. But where an amino acid subject to degradation did undergo interconversion with other amino acids, the extent of this interconversion could not be accurately assessed, since it could not be assumed that all the degradation products were shown on the autoradiograph.

RESULTS

These are set out in the Table. The amino acids used by the connective tissue cell for the synthesis of protein under the present conditions may be divided into two groups: those having carbon skeletons which are in some measure synthesized by the cell, as evidenced by the presence in the amino acids of carbon derived from glucose, and those having carbon skeletons which are not synthesized by the cell. The first group includes glutamic acid, aspartic acid, alanine, proline, threonine, serine and glycine. Glutamic acid, aspartic acid and alanine, as might be expected from their close relationship to intermediates of the tricarboxylic acid cycle, undergo a good deal of interconversion. Each of these three amino acids contributes some carbon to the other two, and to proline; glutamic acid

TABLE—*Radioactivity in each Amino Acid Expressed as a Percentage of the Total Radioactivity in the Whole Protein Hydrolysate*

Labelled compound added to medium	Glut.	Asp.	Ala.	Pro.	Thr.	Ser.	Gly.	Leu +Ileu.	Val.	Phe.	Tyr.	Arg.	Lys.	His.	Try.	Met.	Cys.
Glucose	30	18	28	2	Tr.	19	2	—	—	—	—	—	—	—	—	—	—
Glutamic acid	72	22	Tr.	4	1	Tr.	Tr.	—	—	—	—	—	—	—	—	—	—
Aspartic acid	22	66	5	2	Tr.	4	Tr.	—	—	—	—	—	—	—	—	—	—
Alanine	9	5	84	1	—	Tr.	Tr.	—	—	—	—	—	—	—	—	—	—
Proline	Tr.	Tr.	—	99	—	—	—	—	—	—	—	—	—	—	—	—	—
Threonine	1	Tr.	Tr.	—	98	Tr.	Tr.	—	—	—	—	—	—	—	—	—	—
Serine	—	—	Tr.	—	Tr.	83	17	—	—	—	—	—	—	—	—	—	—
Glycine	—	—	—	—	—	4	96	—	—	—	—	—	—	—	—	—	—
Leucine	2	1	—	Tr.	—	—	—	96	—	—	—	—	—	—	—	—	—
Iso-leucine	2	2	—	Tr.	—	—	—	95	—	—	—	—	—	—	—	—	—
Valine	Tr.	Tr.	—	Tr.	—	—	Tr.	—	99	—	—	—	—	—	—	—	—
Phenylalanine	Tr.	Tr.	—	—	Tr.	—	—	—	—	96	3[1]	—	—	—	—	—	—
Tyrosine	—	—	—	—	—	—	—	—	—	—	100[2]	—	—	—	—	—	—
Arginine	Tr.	Tr.	—	Tr.	—	—	Tr.	—	—	—	—	99	—	—	—	—	—
Lysine	—	—	—	—	—	—	—	—	—	—	—	—	100	—	—	—	—
Histidine[0]	—	—	—	—	—	—	—	—	—	—	—	—	—	100[3]	—	—	—
Tryptophan[0]	—	—	—	—	—	—	—	—	—	—	—	—	—	—	100[4]	—	—
Methionine-^{35}S	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	100[5]	—
Cystine-^{35}S	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	100[6]

Tr. = less than 0·5 per cent. — = no radioactivity detected. [0] = not uniformly labelled.

[1] Some degradation of tyrosine occurred during hydrolysis. The figure given represents the total radioactivity found in tyrosine and those of its degradation products which appeared on the autoradiograph.

[2] All the radioactivity was in tyrosine and its degradation products.

[3] All the radioactivity was in histidine and its degradation products.

[4] A small amount of radioactivity appeared in glycine; but since some labelled glycine was produced by degradation of the tryptophan during hydrolysis, the radioactivity inthe glycine on the chromatogram was regarded as an artefact. This view was supported by the fact that no radioactivity was found in any other amino acid.

[5] Radioactivity from methionine-^{35}S appeared in a number of degradation products. Two of these were methionine sulphone and homocysteic acid; the others were not identified. No radioactivity from methionine-^{35}S appeared in cystine or cysteic acid.

[6] Radioactivity from cystine-^{35}S appeared mainly in cystine and cysteic acid. No radioactivity from cystine-^{35}S appeared in methionine or any of its degradation products.

and aspartic acid contribute carbon also to threonine. Proline and threonine undergo very little conversion into other amino acids; traces of carbon from proline appear in glutamic and aspartic acids, and from threonine in glutamic acid, aspartic acid, alanine, serine and glycine. Serine and glycine undergo interconversion, which is more marked from serine to glycine than from glycine to serine. Both receive small amounts of carbon from glutamic acid, aspartic acid, alanine and threonine, but more, directly from glucose. Glycine contributes no detectable amount of carbon to any other amino acid, and serine contributes traces only to threonine and alanine.

There is no evidence that any of the other amino acids (leucine, isoleucine, valine, phenylalanine, tyrosine, methionine, cystine, arginine, histidine, lysine and tryptophan) is synthesized by the cell under the present conditions; nor do these amino acids undergo any detectable interconversion, except phenylalanine and tyrosine. Some phenylalanine is converted to tyrosine, but tyrosine is not converted to phenylalanine. Methionine is not converted to cystine, or cystine to methionine. This is in keeping with the results of previous work on the sulphur requirement of rat connective tissue cells (Harris, 1955*b*). Small amounts of carbon from amino acids which are not synthesized by the cell appear in some

of the amino acids which are. This is presumably due to some degradation of the former and entry of the degradation products into the general metabolic pool.

DISCUSSION

Some of the findings above are not what might have been predicted from the information available from studies on the whole animal. For example, cysteine is not an " essential " amino acid for the rat if methionine is available, because the rat can convert methionine to cysteine (du Vigneaud, 1952). But the connective tissue cell of the rat does not convert methionine to cysteine, and methionine cannot meet the requirement of the cell for cysteine (Harris, 1955*b*). Threonine is an " essential " amino acid for the rat, but some synthesis of the carbon skeleton of threonine is accomplished by the rat connective tissue cell. On the other hand, arginine can be synthesized by the rat, although not always in amounts sufficient for optimal growth, but the rat connective tissue cell does not synthesize arginine at all.

Differences of this sort illustrate the error of assuming that nutritional requirements determined for the whole animal are necessarily shared by all the cells of that animal. Certain compounds may be produced by reactions which are carried out by special tissues. For example, the synthesis of arginine and the conversion of methionine to cysteine, reactions which do not occur in the connective tissue cell, have been shown to occur in the liver. As cell " lines " derived from various animal tissues are becoming available in increasing numbers, studies like the present one should now be possible on a wide range of animal cells. It will be of interest to see in what way the pattern of protein synthesis differs in different cells and what features are common to all cells.

SUMMARY

The synthesis of protein was studied in a homogeneous population of rat connective tissue cells multiplying *in vitro*. The amino acids used in the synthesis of protein could be divided into those with carbon skeletons in some measure synthesized by the cell, and those with carbon skeletons not synthesized by the cell. The first group comprised glutamic acid, aspartic acid, alanine, proline, threonine, serine, and glycine ; the second comprised leucine, isoleucine, valine, phenylalanine, tyrosine, methionine, cystine, arginine, histidine, lysine and tryptophan. A good deal of interconversion occurred between members of the first group ; little or none occurred between members of the second. The relevance of these findings to studies on the amino acid requirements of the whole animal is discussed.

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THE EFFECT OF SOME FATTY ACIDS AND PHOSPHOLIPIDS ON BLOOD COAGULATION

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It has been reported that phosphatidyl ethanolamine (P.E.) (Robinson and Poole, 1956) or fatty acids (Poole, 1955) can accelerate the clotting time of plasma in various coagulation systems. O'Brien (1956*a*) suggested that platelets are active in blood coagulation because they contain P.E. or some similar substance. He also suggested (1956*b*) that the acceleration of blood coagulation after a fatty meal might be due to an increase in " available " P.E. or a similar substance in the plasma.

It is, therefore, important to know if P.E. is the only phospholipid which can replace platelets and cause acceleration of coagulation similar to that observed after a fatty meal. This paper reports experiments which show that a number of other phospholipids are active in some coagulation systems. Thus, any one of a number of phospholipids or a mixture of them might be responsible for the activity of platelets and the increased activity of lipaemic plasma.

It has been suspected for a long time that a lipoid substance in platelets was essential for normal coagulation. Bordet and Delange (1913) showed that cytozyme, a lipoid extractable from platelets etc., was essential to their clotting system. They also showed that a commercial " lecithin " could replace cytozyme. Wadsworth, Maltaner and Maltaner (1936) showed that " cephalin " produced marked acceleration when introduced into the same system. They produced several new concepts applicable to the present problem. They showed that " cephalin "—which promotes rapid coagulation—will combine stoichometrically with calcium chloride and that lecithin, which is inactive, will not. They studied four systems : (1) The precipitation of serum in the presence of calcium chloride and cephalin, (2) the clotting system of Bordet and Delange (1913), (3) the clotting time of oxalated plasma and (4) the complement system. In all of these, they found that the proportion of the three reactants, protein, cephalin and calcium, were all critical. If any reactant was increased or decreased, then the reactions were inhibited. Using the same sample of " cephalin ", now twenty years old and shown to be pure phosphatidyl serine, Rapport (1956) has expanded this picture. He showed that phosphatidyl serine by itself was only moderately active in shortening the clotting time of his system. If it was dissolved in a fat solvent with two volumes of lecithin, itself inactive, and the mixture was dried and suspended in water, then the activity of the phosphatidyl serine was considerably enhanced. A tenfold addition of lecithin was inhibitory ; so he argued that this increased activity indicated a specific lipid-lipid interaction and was not simply the physical effect of lecithin increasing the solubility of the phosphatidyl serine through mixed micelle formation. Chargaff (1944) also emphasized the

complexity of this work. Starting with impure fractions that were highly active, he found that the more they were purified beyond a certain point, the less active they became. Using the acceleration of the clotting time of rooster plasma, he found phosphatidyl serine inactive and P.E. only moderately active, while impure fractions were highly active. Turner, Silver and Tocantins (1956), in their attempt to identify the chemical nature of their lipid antithromboplastin, find that purification beyond a certain point does not increase the activity of the fraction; phosphatidyl serine, however, seemed to be a major component.

In England, Trevan and Macfarlane (1936) showed that crude commercial "lecithin" greatly accelerated the clotting of human plasma by Russell viper venom (the Stypven time). However, pure lecithin is inactive (Poole, Robinson and Macfarlane, 1955). Using platelet-poor rat plasma, Poole and Robinson, (1956) and Robinson and Poole (1956) found that P.E., which is probably a contaminant of commercial "lecithin", accelerated the Stypven time and increased the thrombin generated, while the serine and inositol phosphatides were almost inactive at a concentration of 1 mg./ml. O'Brien (1956*a*) confirmed and investigated further the activity of P.E.: the purest P.E. obtained from egg was considerably less active than a slightly less pure sample from brain. Maclagan and Billimoria (1956) have shown that a fraction isolated from butter which contains P.E. and galactose is an accelerator of the Stypven time of human plasma. Wallach, Surgenor and Walters (1956) have shown that platelets contain P.E. and phosphatidyl inositide, but have not isolated and tested these substances in clotting systems.

There is less information about the action of free fatty acids on blood coagulation. Poole (1955) reported that the sodium salts of some fatty acids produced moderate acceleration of the Stypven time of platelet-poor plasma. Laurate, oleate and linoleate were active, while palmitate and stearate and other fatty acid salts were inactive. In a thrombin generation test in which the plasma was already moderately active in producing thrombin, the addition of sodium laurate and sodium stearate produced increased activity. The greatest effect was produced by a concentration of 3·3 m-equiv./l but activity could be shown by as little as 20μe-quiv./l.

MATERIALS AND METHODS

Each substance to be tested was added in a range of concentrations to a substrate of non-lipaemic platelet-poor human plasma and the effect was compared with a negative control containing saline in place of the test substance, and a positive control containing an optimal number of platelets. Previous experiments had shown that Asolectin, a crude commercial product, simulated the action of platelets in all respects and, in practice, Asolectin (1 mg./ml. normal saline) was usually substituted for platelets.

The substrate for the tests was citrated platelet-poor non-lipaemic human plasma. This was prepared by collecting human blood after a fat-poor breakfast. A silicone technique was used throughout. Sodium citrate was added to the blood in the proportion of 1 part 0·025 M citrate to 9 parts blood and the mixture centrifuged at once for 15 min. at 3500 r.p.m. All experiments were carried out at 37° and the results are expressed as the average of at least two separate tests performed on the same day. All test substances were made up at a concentration of 1 mg./ml. (or stronger, if supplies allowed) and serial tenfold dilutions in distilled water were prepared therefrom. In each test, sufficient dilutions were used to ensure that the optimum concentration had been passed in both directions, or that a plateau of maximal activity had been reached. In all tests the positive and negative controls were carried out at the same time; the positive control consisted of substituting platelets or Asolectin for the test substance; in the negative control saline was substituted.

The clotting time.—Plasma, 0·1 ml., was pipetted into each of four small glass test tubes together with 0·1 ml. of the substance to be tested. One minute later, 0·1 ml. of 0·025 M-$CaCl_2$ was added. The clotting time is recorded as the average of the four determinations.

Prothrombin consumption.—Following the determination of the clotting time the serum from the four tubes was pooled and tested by the method of Biggs and Macfarlane (1953).

Thrombin generation.—The substrate plasma was recalcified after adding the test substance and at suitable intervals thereafter subsamples were transferred to tubes containing a fibrinogen solution, and the clotting times of the fibrinogen subsample tubes were recorded. The speed of clotting of the fibrinogen is determined by the amount of thrombin present in the transferred subsample. The result reported is the shortest clotting time in sec. of any of the subsample tubes. This is a measure of the max. concn. of thrombin that occurred during the coagulation of the original plasma : the shorter the reported time, the higher is the peak concentration of thrombin in the orginal plasma (for further details see O'Brien, 1955).

Thromboplastin generation test.—The method of Biggs and Douglas (1953) was used, and the figure reported is the shortest clotting time in sec. in any subsample tube.

Stypven test.—To 0·1 ml. of plasma was added 0·1 ml. of a suspension of the material under test and 0·1 ml. of 1/10,000 dilution of Russell viper venom (Stypven Burroughs Wellcome and Co.) in distilled water ; 1 min. later, 0·1 ml. of 0·025 M-$CaCl_2$ was added and the time then taken for clotting to occur was recorded in sec.

Materials.—Egg lecithin (Sylvana), prepared by the method of Pangborn (1951), may be assumed to be almost pure and free of acetal. It was shown to contain less than 1 per cent P.E. and less than 2 per cent inositides. Egg lecithin, prepared on silica columns by G. B. Robinson, was similarly pure. Beef heart lecithin (Sylvana), prepared by the Pangborn method (1951), contains, as well as lecithin, the acetal or plasmalogen substance called phosphatidal choline (Hack, 1953) which may represent 60 per cent of the total (Rapport and Alonzo, 1955). Cardiolipin (Sylvana), probably has the formula suggested by Pangborn (1947) and was prepared by her method (1942). It was investigated since it has a close chemical relationship to phosphatidic acid. The egg lecithin-cardiolipin mixture was prepared by mixing equal parts of these two substances in alcohol. The alcohol was removed by a stream of warm air and the dry residue was suspended in distilled water. The phosphatidyl ethanolamine (P.E.) used was pure, but was at the time of use of a middle brown colour. The acetal of P.E., phosphatidal ethanolamine was contaminated with 33 per cent P.E. It was a brown powder. The pure P.E. was separated from this mixture. Phosphatidyl serine (Klenk), was a brown powder, containing 20 to 40 per cent P.E. L-α-phosphatidyl serine (Hawthorne), prepared from brain, may contain small quantities of P.E. and inositol phosphatide. For the phosphatidyl serine (Wadsworth *et al.*, 1936) used Rapport, who kindly gave me a part of Wadsworth's original preparation, produced evidence that this sample was pure (Rapport, 1956). The brain diphosphoinositide with the probable formula suggested by Hawthorne (1955) was certainly impure. It may have contained 40 per cent of phosphatidyl serine but did not contain P.E. or lecithin. Liver phosphatidyl inositide probably has the formula of Faure and Morelec-Coulon (1953). The sample was contaminated by phosphatidyl serine but not by P.E. Sphingomyelin was over 98 per cent pure, the only possible contaminant being a trace of cerebroside. Since the Sphingosine and the sphingosine hydrochloride preparations proved to be inactive, the question of their purity, and therefore, a possible content of an active impurity did not arise. Asolectin is a crude commercial product prepared from soya " lecithin "containing about 95 phosphatides, including 30 per cent lecithin, 30 per cent cephalin and 35 per cent " lipositol "—mostly inositides.

RESULTS

The optimal results of from one to six tests for each substance on different plasmas were averaged irrespective of the dilution of the substance used in each test. This average time and that of the positive and negative control for all the clotting tests are presented in Table I. The times for the inactive substances are omitted. Inactivity (—) means that the plasma containing the test substance behaves in the same way as the negative control. Maximal activity, indicated by +++, is defined as an effect by the test substance equivalent to that pro-

TABLE I.—*Activity of Some Phospholipids Compared with Platelets and Saline*

Test substance	Clotting time in min.			Thrombin generation in sec.			Serum prothrombin time in sec.			Thromboplastin generation in sec.			Average activity in the foregoing tests	Stypven time in sec.			
	Saline	Platelets	Test substance	Saline	Platelets	Test substance	Saline	Platelets	Test substance	Saline	Platelets	Test substance		Saline	Platelets	Test substance	Activity
Beef lecithin	5¼	1¼	5	120	21	120	35	>90	65	34	9	60	−	20	5	8	++
Cardiolipin	6	1¼	6	140	21	146	34	>90	40	34	9	37	−	19	5½	11½	+
Mixture of lecithin and cardiolipin	5½	1½	4¾	85	24	86	27	>90	32	33	8	44	−	17	6	6	+++
Phosphatidyl ethanolamine (P.E.)	4	1	1¾	>120	27	26	30	>90	>90	28	10	9	+++	32	5½	5	++++
Phosphatidal ethanolamine	4	1	1¾	>120	16	14	30	>90	>90	28	10	11	+++	32	5½	5	++++
Phosphatidyl serine (Klenk)	4	1	1	>120	16	13	30	>90	>90	28	10	10	+++	32	5½	5½	++++
Phosphatidyl serine (Hawthorne)	5	1¾	4	>120	23	49	38	>90	>90	37	9	20	+	23	4¾	10	++
Phosphatidyl serine (Wadsworth)	5½	1¼	3½	80	25	58	26	>90	>90	30	7¼	18	+	18	5½	6	++
Liver phosphatidyl inositide	5¾	1	2½	95	31	34	27	>90	>90	33	8	19	++	23	4¾	9	++
Brain diphosphoinositide	6	1	1½	90	31	27	26	>90	>90	33	8	9½	+++	19	5	5	+++

Sodium glycerophosphate
Egg lecithin (two sources)
Sphingomyelin
Sphingosine hydrochloride
Sphingosine
} Completely inactive in all tests.

duced in the positive control by the suspension of platelets or Asolectin. Intermediate degrees of activity are indicated by : ++, +, and ±.

The following substances were found to be completely inactive, or slightly inhibitory : egg lecithin (two sources), sphingomyelin, sphingosine hydrochloride, sphingosine, and sodium glycerophosphate. Although egg lecithin was completely inactive, the beef lecithin, containing a considerable proportion of acetals, was moderately active in the Stypven clotting system, but inactive in all the other test systems. Cardiolipin behaved similarly to the lecithin acetal, having a moderate effect only on the Stypven time ; the mixture of cardiolipin and the inactive egg lecithin had maximal activity in the Stypven test, *i.e.*, enhanced activity, but was inactive in the other tests. Two samples of phosphatidyl serine were both slightly active in all the tests not involving Stypven and were moderately active in the Stypven test. The third sample of phosphatidyl serine was fully active in all tests. The liver phosphatidyl inositide was moderately active in all tests while the brain diphosphoinositide was fully active.

The fully active and the inactive substances were consistent in their behaviour, but this was not so with the intermediate substances. Table II gives two examples.

TABLE II.—*The Variability of the Effect of Phospholipids in Coagulation Tests*

	Thrombin generation (sec.)				Stypven time (sec.)		
Test substance	Saline	Platelets	Test substance	Test substance	Saline	Platelets	Test substance
Phosphatidyl serine (Hawthorne)	70	25	80	Cardiolipin	16	6	16
	105	21	21		22	5	7
	>120	22	60		18	4½	12
	78	13	24		—	—	—
	>120	26	34		—	—	—
	>120	25	60		—	—	—
	>120	26	63		—	—	—
Average	120	23	49	Average	19	5½	11½

Details of some individual experiments averaged in Fig. 1.

Had stronger concentrations of all the substances been available, they would probably all have had an inhibitory effect at high concentration. Brain inositide showed no evidence of inhibition at a concentration of 1 mg./ml. but all the other substances showed some evidence of inhibition at this strength, either in the dilute system used in the thromboplastin generation test or in the Stypven test, and often in both.

Slightly different types of inhibition and acceleration were encountered. In the Figure the Stypven times of fasting platelet-poor plasma containing logarithmically increasing amounts of phospholipids are plotted against the concentration of the phospholipid added. It will be seen that Asolectin produced maximal acceleration (*i.e.*, as good as platelets) over a considerable range. Beef heart lecithin on one occasion produced maximal acceleration but at one concentration only, and any increase in concentration thereafter produced inhibition. On another occasion, using different plasma, the beef heart lecithin behaved quite differently. A plateau of optimal activity was reached at 11 sec. and further

increments only produced inhibition. Two samples of phosphatidyl serine regularly produced inhibition in high concentration and, as will be seen, there is a critical concentration which gives the optimal acceleration of the Stypven test. The variability of this optimal effect has already been stressed in Table II. Egg lecithin is inactive at all concentrations used.

The minimum concentration necessary to produce demonstrable acceleration of the Stypven time was about 0·1 to 1·0 μg. per ml. This concentration was about

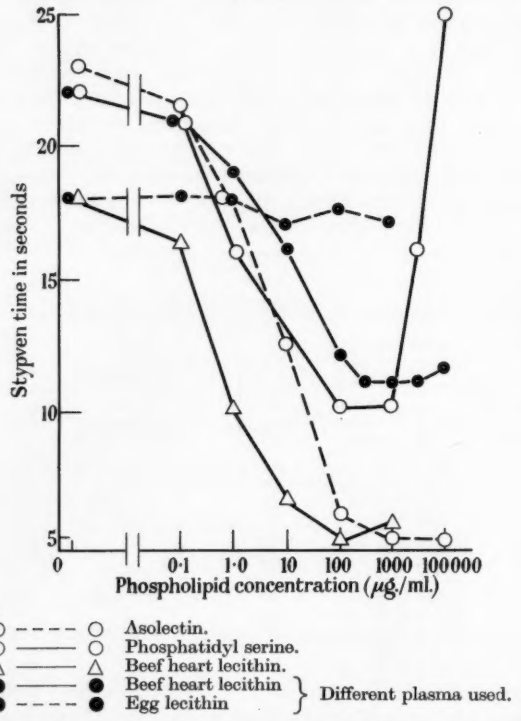

FIGURE.—The effect on the clotting time of the concentration of phospholipids added to platelet-poor plasma and Russell's viper venom.

To 0·1 ml. of platelet-poor plasma and 0·1 ml. Russell's viper venom, 1/10,000, was added 0·1 ml. of the phospholipid at the concentration indicated. One min. later 0·1 ml. 0·025 M-$CaCl_2$ was added and the clotting time recorded: each point is the average of at least two determinations.

the same for all substances showing any activity, and was independent of the degree of activity shown at optimal concentration.

Table III summarizes the results of introducing optimal concentrations of the sodium salts of three fatty acids into various clotting systems. It will be seen that sodium laurate was moderately active in the Stypven test but inactive in all the others. Sodium oleate was moderately active in the Stypven test and sodium stearate slightly so. Sodium oleate and stearate repeatedly produced

some shortening of the clotting time of plasma, but were virtually inactive in the other tests.

TABLE III.—*Activity of Fatty Acids in Various Clotting Tests*

Test substance	Clotting time (min.)			Thrombin generation (sec.)			Serum prothrombin time (sec.)			Average activity in foregoing tests	Stypven time (sec.)			
	Saline	Platelets	Test substance	Saline	Platelets	Test substance	Saline	Platelets	Test substance		Saline	Platelets	Test substance	Activity
Sodium laurate	4¼	1¾	4½	120	24	110	22	>120	21	—	33	5	10	++
Sodium oleate	4¼	1¾	2¾	160	30	73	—	—	—	±	18	4½	9	++
Sodium stearate	4¼	1¾	2¾	120	24	120	22	>120	23	±	33	5	21	+

DISCUSSION

The variability of the results using substances of moderate activity is emphasized in the Figure and in Table II. This variability may be due in part to the difficulty of getting the substances, all insoluble in water, into a suitable state of dispersion. It was found, however, that even when the same suspension was tested day after day with different plasmas, some, but less, variation persisted. When the clotting time of plasma with saline added (the negative control) was long, the test substance usually gave a long clotting time, although the maximal acceleration with platelets (the positive control) was constant. This suggests that the Russell viper venom was probably behaving consistently; but the presence of lipids or other substances in the plasmas, which were all prepared identically, may have influenced the effect of the added phospholipid.

Rapport (1956) has shown that the activity of phosphatidyl serine in a different clotting test is increased by dissolving it in a fat solvent with lecithin. It has now been shown that the mixture of cardiolipin and lecithin is more active than cardiolipin by itself. These two observations support the general proposition that the action of a particular phospholipid may be modified by the presence of other phospholipids, as for example, in the Wassermann Reaction. Wadsworth *et al.* (1936) have shown that behaviour of phospholipids may depend on the relative concentration of these substances to protein and to calcium ions.

The variability of the results reported here does not represent technical ineptitude, but emphasizes an important aspect of the behaviour of phospholipids in biological systems. Without more knowledge of the relevant factors it is impossible to define accurately the degree of activity of each phospholipid, since the activity may depend on unknown factors in the plasma.

Since P.E. is known to be active, it might be argued that all these substances were active by virtue of their contamination by P.E., but it can be shown that this is not true. Wadsworth's sample of phosphatidyl serine, which was pure according to Rapport (1956), was found to be moderately active in the present investigation. The greater activity of Professor Klenk's sample might be due to degradation products, since it was middle-brown in colour while the other two samples were cream coloured. The phosphatidyl serine preparation of Hawthorne certainly

contained some P.E., but these inositide preparations contained none (although they contained phosphatidyl serine).

The interpretation of the results with P.E. and the acetal of P.E. are more difficult. Both were brown and so might have contained active degradation products. However, the preparation containing 66 per cent acetal and 33 per cent P.E. was weight for weight at all dilutions as active as the pure P.E. It is therefore probable that the acetal was itself active or enhanced the activity of the P.E.

The active phospholipids including the pure phosphatidyl serine all showed some activity in a concentration of about 1 μg./ml. It seems impossible that at this high dilution they were all active by virtue of an impurity. The observation that the minimum concentration showing detectable activity was approximately the same for all the active phospholipids irrespective of the degree of activity shown at optimal concentration is curious, but at present unexplained.

Poole (1955) reported that sodium stearate increased the amount of thrombin generated in a test system where a considerable quantity of thrombin was being generated by the plasma. In the present work, when the plasma alone generated almost negligible quantities of thrombin, the addition of sodium laurate and stearate in all concentrations repeatedly failed to increase the quantity of thrombin generated ; the results with the Stypven test are in complete agreement with those of Poole.

The present results do not support the suggestion that the clotting changes after a fatty meal are due to an increase in free fatty acid in the plasma (Poole, 1955). O'Brien (1955) claimed that after a fatty meal there was a slight increase in the thrombin generated by platelet-poor plasma. If the present observation that fatty acids do not affect thrombin generation in plasma can be confirmed, then it is most unlikely that the increase in unesterified fatty acids is solely responsible for all the coagulation changes observed after a fatty meal.

Gordon and Cherkes (1956) reported that the unesterified fatty acids increase in the plasma after a fatty meal, and that they also increase after a twelve hour period of starvation, but there is no increase after a carbohydrate meal. No coagulation changes have been described in starvation or after a carbohydrate meal.

O'Brien (1956*a*) has shown that P.E. can simulate the action of platelets in blood coagulation, and therefore might be the " active " substance in platelets. It is now seen that phosphatidyl serine, phosphoinositide and diphosphoinositide which also may be present in platelets, may play a part in normal blood coagulation ; and there is no evidence that the list is complete. It is even possible that the appearance or increase in a substance like lecithin which is inactive alone, might potentiate other active phospholipids previously present but in small and inactive quantities.

The increase of free fatty acids after a fatty meal may possibly be responsible for the post-prandial changes in blood coagulation. On the other hand, an increase in active phospholipids in the plasma post-prandially seems more likely to play a major part. Further evidence in support of this hypothesis is adduced elsewhere (O'Brien, 1957). The present work suggests that the observed results in lipaemic plasma could be produced by an increase in the plasma of free available diphosphoinositide, phosphatidylinositide, phosphatidyl serine or P.E. Furthermore, it has already been shown (O'Brien, 1956*b*) that in some tests the P.E.-

like substance in platelets is not immediately available. P.E. is essential for blood coagulation *in vitro*, but presumably only becomes available during the process of blood clotting after the platelets have been " activated ". Thus, a small amount of free active phospholipid might have a disproportionate effect on blood coagulation by short-circuiting this initial activating stage involving the platelets.

It has previously been tentatively assumed that in experiments on lipaemic plasma the highly reproducible Stypven time and the plasma clotting time, notoriously susceptible to minute changes in technique, were, in fact, measuring the same phenomenon ; and that is why the Stypven time has sometimes been preferred in such investigations (O'Brien, 1956*b*, 1957). This assumption was based on the observation that the two tests varied roughly in parallel (O'Brien, 1955). It is now shown that cardiolipin, the acetal of lecithin, and some free fatty acids all accelerate the Stypven time but do not affect the plasma clotting time or thrombin generation. The above assumption is thus not always justified.

The degree of activity of some phospholipids in various clotting systems probably depends on the presence of other lipids in the plasma and other unknown variables. The optimal concentration of such phospholipids is often critical and most of them show inhibition when present in excess.

O'Brien (1956*a*) has previously shown that the lyso-compound of P.E. is inactive. It is now shown that, while lecithin is completely inactive, the acetal of lecithin is active in the Stypven test and the acetal of P.E. is probably active in many systems. Thus, the arrangement of the fatty acids in the α- and β-positions of the glycerol molecule is also important. On the other hand, the presence of double bonds in the fatty acids may be unimportant since O'Brien (1956*a*) did not detect much difference in activity in the natural unsaturated P.E. and a hydrogenated P.E.

The chemical properties of sphingomyelin and lecithin, which are inactive in coagulation systems, differ from those of P.E., phosphatidyl serine and phosphatidyl inositide, which are active. With their quaternary ammonium group, they have a stronger positive charge and are almost neutral at physiological pH. Unlike lecithin and sphingomyelin the more basic cephalins can combine with calcium and also form salts with tissue proteins (Wadsworth *et al.*, 1936; Chargaff, 1946). These properties may be related to their action in blood coagulation.

SUMMARY

Using the plasma clotting time, the thrombin generation test, the prothrombin consumption test, the thromboplastin generation test and the Russell viper venom accelerated clotting time (Stypven time), it has been shown that the diphosphoinositide, phosphatidyl inositide, phosphatidyl serine and the acetal of phosphatidyl ethanolamine (phosphatidal ethanolamine) can to varying extents mimic the action of platelets. It has previously been shown that phosphatidyl ethanolamine can also substitute for platelets. Some fatty acids accelerate the plasma clotting time and stypen time only. Cardiolipin and the acetal of lecithin accelerate the Stypven time only. Egg lecithin, sphingosine and sphingomyelin are inactive. The activity of one phospholipid may be influenced by the presence of another inactive phospholipid.

It is concluded that platelets may be active in *in vitro* clotting systems by virtue of their content of any one of a number of phospholipids. The coagulation

changes observed in platelet-poor plasma after a fatty meal are not likely to be due to an increase of unesterified fatty acids, but could be fully explained if there was an increase in the plasma of any one of several, or a combined increase in several free and available phospholipids.

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SEVEN VIRUSES ISOLATED FROM THE VERVET MONKEY

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THE vervet monkey, *Cercopithecus aethiops pygerythrus*, is the animal most commonly used in South Africa for the laboratory isolation and maintenance of strains of poliomyelitis virus, and for the production of poliomyelitis vaccine. Following the introduction by Enders, Weller and Robbins (1949) of cultures of non-nervous tissue for the growth of this virus, various tissues from the vervet monkey have been used for this purpose, the most suitable tissue for large-scale cultures, and also the most sensitive for the isolation of poliomyelitis and a number of other human viruses, being renal epithelium prepared according to the trypsinization technique of Youngner (1954).

It is known that viruses may accompany tissues cultivated *in vitro*, manifesting themselves after variable periods in the cultures. The first of the group of agents now classified as adenoviruses was recognized through the occurrence of spontaneous degeneration in cultures of human adenoids (Rowe, Huebner, Gilmore, Parrott and Ward, 1953), and workers in the field of poliomyelitis research have isolated simian viruses from cultures of rhesus and cynomolgus kidney tissue (Hull, Minner and Smith, 1956). It is the object of this paper to describe briefly the effects in tissue culture of seven simian viruses which have been encountered since 1954 in the laboratories of the South African Poliomyelitis Research Foundation. Six of these viruses were isolated from uninoculated cultures of renal tissue from the vervet monkey, and one from the faeces.

Until a standardized classification of simian viruses has been adopted, it is proposed to call this particular group the SA viruses ; and the following is a brief account of their behaviour in tissue culture.

SA1 (Fig. 1) produces syncytia with aggregates of nuclei of normal appearance, and is possibly identical with the MK virus described by Rustigian, Johnston and Reihart (1955). Vacuolation is frequently absent, and it is not yet known whether this represents a true difference between strains. It is the virus most commonly encountered in kidney cultures, usually appearing 10 to 14 days after preparation of the culture, the period being considerably shortened with passage. Syncytia arise in multiple foci, later coalescing to involve the entire culture which then flakes from the glass.

SA2 (Fig. 2) which has been isolated on several occasions from monkey kidney, produces intranuclear inclusions in syncytia. After several passages the inclusions tend to disappear, leaving syncytia with abnormal chromatin patterns. This agent does not have the characteristics of herpes B virus.

SA3 (Fig. 3) has been recovered frequently from kidney cultures. Cytopathic effects appear in foci about 10 to 14 days after the preparation of cultures, extending to involve the whole tissue over a period of days on initial isolation. The cells,

which show a fine refractile granularity, develop elongated processes in a reticular pattern before they fall off the glass. Staining with haematoxylin and eosin demonstrates large red cytoplasmic inclusions. These commence at multiple points in the cell, increasing in size and fusing to form homogeneous masses which lie around and over the nucleus. Similar inclusions are formed in cultures of HeLa cells and human amnion. This agent, which is clearly of simian origin, is neutralized by an antiserum, kindly supplied by Dr. A. B. Sabin, against the human ECHO 10 virus. Cytoplasmic inclusions identical in appearance with those produced by SA3 are observed in monkey kidney cells infected with ECHO 10 virus.

SA4 (Fig. 4) is more rapid in its cytopathic effect which somewhat resembles that produced by poliomyelitis virus. In preparations stained with haematoxylin and eosin, the characteristic features are margination of chromatin with an eosinophilic even appearance of the central part of the nucleus, followed by the formation of a large pale paranuclear area and lateral displacement of the nucleus which becomes crumpled or scrolled. Two eosinophilic dots are frequently seen in the open ends of the scrolled nucleus. Changes somewhat similar have been described by Reissig, Howes and Melnick (1956) in cells infected with poliomyelitis virus, but pallor of the nucleus followed by scrolling is more often seen in cells infected with SA4. This agent has been isolated on numerous occasions from kidney cultures.

SA5 produces a cytopathic effect similar to that of SA4, but is not neutralized by the serum of monkeys naturally immune to SA4.

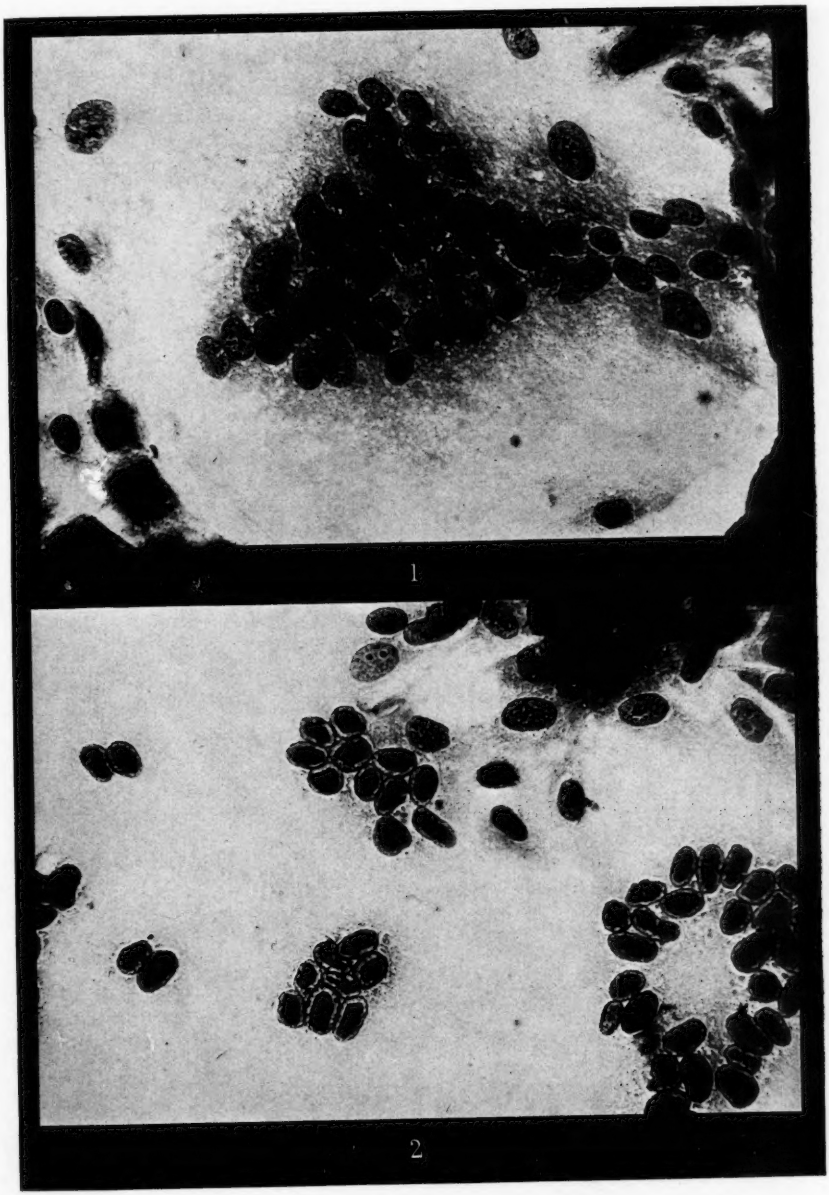
SA6 (Fig. 5) has been recovered on three occasions from kidney cultures. Foci of rounded swollen cells appear after 14 days or more, progressing in size only very slowly, and passage does not markedly increase the rate of cell destruction. When stained with haematoxylin and eosin, the cells show lateral displacement and elongation of the nucleus which has, characteristically, either two small inclusions or one long inclusion. Binucleate masses are common, and there is increased phagocytosis of one cell by another. Fig. 5 shows a small focus with typical changes.

SA7 (Fig. 6) was isolated from monkey faeces in the course of a poliomyelitis virus feeding experiment. Growth in kidney epithelium is more rapid than in the case of SA6, and the nuclear inclusions are distinctive. Inclusions are formed in an annular pattern around the nucleolus, and can be seen to be multiple, with concentric structures. These later fuse into a homogeneous dense mass which is round or oval, being less elongated than that produced by SA6.

The majority of cultures in which these agents have been isolated consisted of trypsin-dispersed kidney epithelium grown at 36°–37° either as stationary cultures in Roux bottles or as roller tubes. The nutrient medium usually comprised Hanks' balanced salt solution containing 0·5 per cent lactalbumen

EXPLANATION OF PLATES

FIG. 1.—Syncytium in monkey kidney epithelium produced by SA1 (× 380).
FIG. 2.—Intranuclear inclusions in syncytia produced in monkey kidney epithelium by SA2 (× 380).
FIG. 3.—Cytoplasmic inclusions produced by SA3 in monkey kidney epithelium (× 960).
FIG. 4.—Monkey kidney epithelial cell infected with SA4 (× 960).
FIG. 5.—Focus of monkey kidney epithelial cells infected with SA6 (× 380).
FIG. 6.—Intranuclear inclusions in a kidney epithelial cell infected with SA7 (× 1200).



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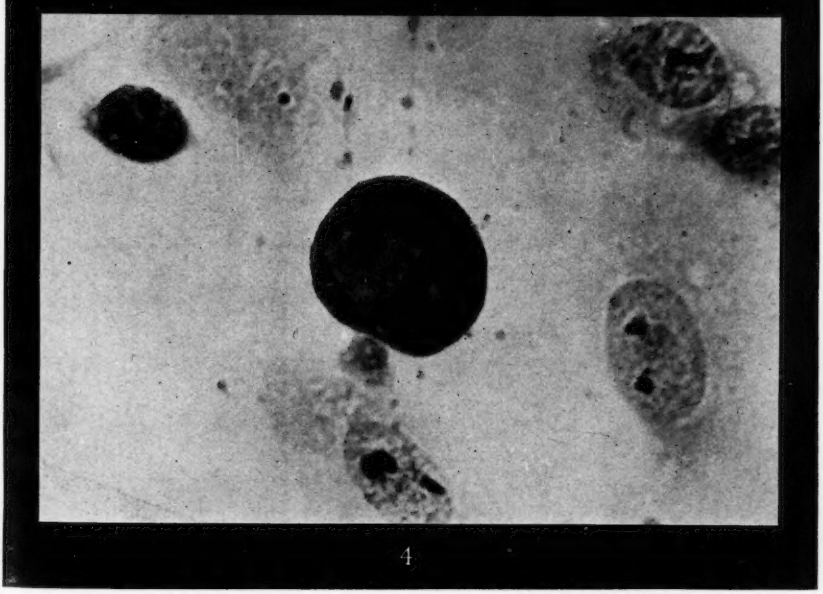

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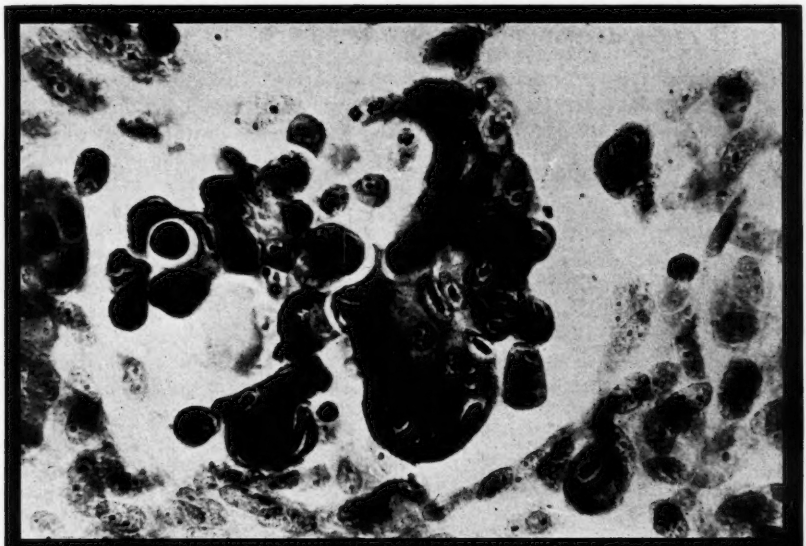

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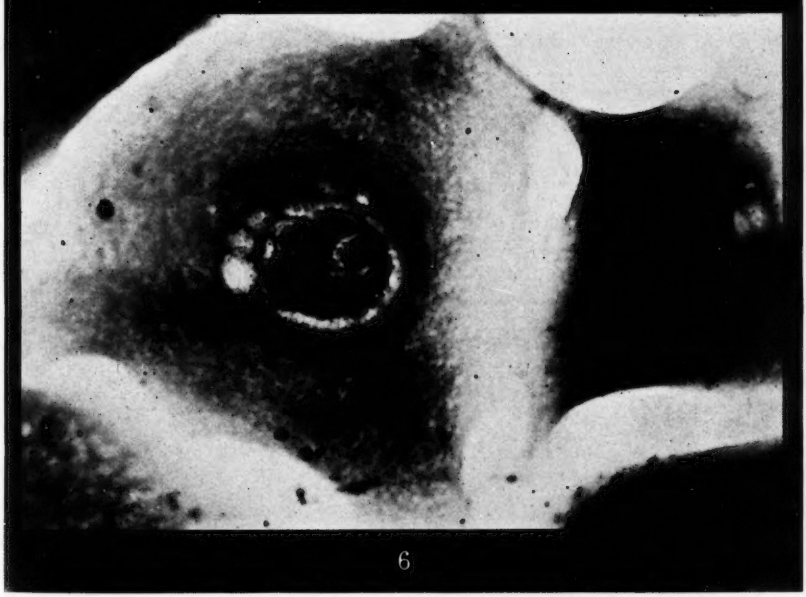

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hydrolysate, with the addition of up to 5 per cent horse serum. The possibility that some of the viruses were derived from either the lactalbumen or the serum has been considered, but is not accepted as likely since their appearance can be clearly seen to follow the use of certain batches of tissue, not batches of serum or lactalbumen. Precautions against contamination during the removal of kidneys make it improbable that the source of virus was not the tissue itself; but it is not possible to ascertain whether virus was present within the cells or in the blood contained in the kidney.

The use, for certain purposes, of small tissue batches consisting of kidneys from one or two monkeys only has led to a marked reduction in the number of isolations of SA3 and SA4, but SA1 continues to be frequently encountered in these small batches. The occurrence of such viruses in tissues used for the isolation of pathogenic agents from human material can be misleading, and an acquaintance with the viral concomitants of any tissue is essential. Inoculation of coverslips lying free in roller tubes, which can be fixed and stained in the tube, provides an additional means of identifying the causes of cellular degeneration, and serves as a useful screening precaution when simian viruses are frequently encountered.

More complete studies of the properties of the SA group of viruses are in progress, but it is felt that a preliminary description of their appearance in tissue culture will be of use, in view of the wide use made of simian tissues for viral cultivation.

SUMMARY

The cytopathic effects in tissue culture of seven viruses recovered from the South African vervet monkey are illustrated and briefly described.

We wish to thank Mr. M. Ulrich, of the Photographic Department of the South African Institute for Medical Research, for the photomicrographs.

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THE ANTIBODY RESPONSE OF MONKEYS, GUINEA-PIGS AND COTTON RATS TO BRITISH POLIOMYELITIS VACCINE

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THE method employed in this country for testing the antigenic activity of formalin-killed poliomyelitis vaccine is similar to that used in America. It involves the immunization of at least 12 rhesus monkeys with three doses of vaccine, each of 1 ml., at intervals of seven days, and the determination of the antibody levels seven days after the third dose. The test has the disadvantage that the cost of the animals and their housing is high, thereby limiting their use in many laboratories and impeding investigations of the most suitable immunization schedule and of the antigenic response to graded doses of vaccine.

In an attempt to produce a more satisfactory test, studies have been made of the antigenic activity of poliomyelitis vaccine in small laboratory animals. It has been shown that both guinea-pigs (Gard, Wesslén, Fagraeus, Svedmyr and Olin, 1956) and cotton rats (Tobin, 1956) are capable of producing specific neutralizing antibodies to all three types of poliomyelitis virus in response to injections of vaccine, and it has been suggested that these animals might be of value in testing vaccine. In view of these observations, it was considered desirable to test in guinea-pigs and cotton rats, as well as in monkeys, the antigenic activity of the three batches of poliomyelitis vaccine used in this country in the 1956 immunization programme. For comparison a batch of American vaccine was also included in the tests. Studies were also made in guinea-pigs of the effect of varying the interval between the doses of vaccine on the antibody response.

MATERIALS AND METHODS

Vaccines.—The three batches of British vaccines, designated 4, 5 and 6, were prepared by the Glaxo Laboratories Ltd. from the virus strains Brunenders (Type 1), MEF-1 (Type 2) and Saukett (Type 3). The batch of American vaccine, E/2769, was prepared by Eli Lilly and Company and differed from the British vaccine in that it contained the Mahoney strain as the type 1 component.

Animals.—Groups of 10 to 15 animals of either sex were used. Rhesus monkeys weighed 1·6–2·8 kg., guinea-pigs 300–350 g. and cotton rats 200–250 g.

Immunization.—In the first series of experiments the animals were immunized with three doses of undiluted vaccine at intervals of seven days and sera were collected seven days after the last injection. For the monkeys, each dose was 1 ml. given intramuscularly and for the guinea-pigs and cotton rats each dose was 0·2 ml. given intradermally as suggested by Gard *et al.* (1956).

In the second series of experiments only guinea-pigs were used. They received two doses, each of 0·2 ml., of undiluted vaccine intradermally and the interval between the doses was varied from 1 to 6 weeks, sera being collected seven days after the second dose.

Collection of sera.—Monkeys were bled from the femoral vein. Guinea-pigs and cotton rats were deeply anaesthetized, the thorax opened and the inferior vena cava cut while the heart was intact before finally lacerating the heart. The volume of blood accumulating in

the thoracic cavity of guinea-pigs and cotton rats was about 15 ml. and 4 ml. respectively, and this was removed by means of a Pasteur pipette. The serum separating from the blood clot was stored at − 10° after inactivation at 56° for 30 min.

Neutralization test.—Sera were titrated by the neutralization method using cytopathic changes in monkey kidney cell cultures as an indication of incomplete neutralization of virus. Serial dilutions of serum in Hanks' balanced salt solution were mixed with an equal volume of virus suspension in Parker 199 solution containing on the average 100 $TCID_{50}$ per 0·25 ml. of the required type of virus, Brunenders, MEF-1 and Saukett being used as the test viruses. After the mixtures had remained at 37° for 3 hr., 0·5 ml. of each was added to 7-day old monkey kidney cell cultures washed with Hanks' solution and fed with 1 ml. of Parker 199 medium containing 0·07 per cent. bovine albumin (Armour), 0·2 per cent sodium bicarbonate and antibiotics. The inoculated monkey kidney cell cultures were rotated at 37° and finally examined for cytopathic changes at seven days.

Two-fold serial dilutions were made of monkey sera and four-fold dilutions of guinea-pig and cotton rat sera. Two cell culture tubes were inoculated with each serum-virus mixture and the highest dilution of serum protecting against the cytopathic effect of virus was taken as the end-point of the titration. Titres are given as the dilution of serum in the serum-virus mixture before addition to cell cultures. The geometric mean titre was calculated for each type for each group of animals.

In each set of tests the British Standard Poliomyelitis Antisera were titrated. These lyophilized sera when re-constituted were kept at 4° and used within seven days since it was found that their antibody titre began to decrease on keeping for longer periods. Two-fold serial dilutions were made of the Standard Sera and four monkey kidney cell cultures used for each serum-virus mixture. Also in each set of tests the virus suspension was titrated, using 10 culture tubes for each virus dilution, and the test dose of virus calculated by the method of Reed and Muench (1938).

It is noted that serum and virus were left in contact for 3 hr. at 37° before adding to cell cultures. Preliminary tests with the British Standard Antisera showed that wide variations in titre were obtained when the mixtures remained at room temperature for 1 hr., as is the usual practice, even though this was followed by a further period of 18 hr. at 4°. More consistent results, however, were obtained when the mixtures were incubated at 37° and even at this temperature it was necessary to leave the mixtures for 3 hr. in order to obtain complete neutralization. During this time no appreciable reduction in the test dose of virus occurred. This method of titration has given reproducible results, as may be seen from Table I, which records the titres obtained in 15 separate titrations of the three British Standard Antisera taken over a period of 12 months during which time 75 titrations were made. The range of titres during this period was 320 to 508 for type 1, 640 to 1690 for type 2 and 640 to 1920 for type 3. The differences in titre were not attributable to the test dose of virus, which ranged from 30 to 370 $TCID_{50}$ in the different tests.

TABLE I.—*Results of Titrations of the British Standard Poliomyelitis Antisera over a Period of* 12 *Months*

Date of test	Antibody titre to :—		
	Type 1	Type 2	Type 3
15.6.56	480	1016	1016
11.7.56	480	1690	1690
13.9.56	440	960	960
31.9.56	508	806	880
3.11.56	508	960	1016
27.11.56	480	960	1280
6.12.56	404	806	806
26.12.56	404	806	960
14.1.57	440	640	960
21.1.57	320	960	960
5.2.57	320	640	1280
12.3.57	320	640	880
17.4.57	480	1280	1280
20.5.57	480	960	1280
11.6.57	480	640	960

RESULTS

Antibody Response in Monkeys, Guinea-pigs and Cotton Rats

The results of the first series of experiments in which the four vaccines were tested in monkeys, guinea-pigs and cotton rats are shown diagramatically in the Figure. Groups of 10 to 12 animals were used. It is evident that, with each species of animal, the batches of vaccine showed a similarity in antigenic activity for each type ; on no occasion was there any striking difference between the batches. In view of this similarity the results with all batches have been grouped together for each species (Table II). These clearly show a wide scatter of titres with each type indicating the large variation in response of individual animals.

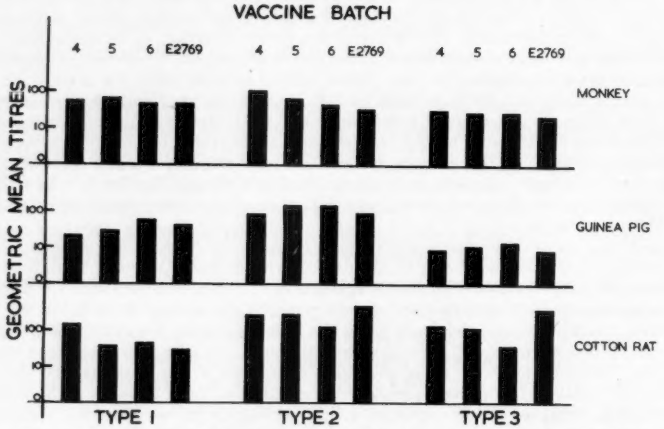

FIGURE.—Geometric mean antibody titres (log. scale) of sera from monkeys, guinea-pigs and cotton rats after immunization with poliomyelitis vaccine.

The variation appears to be greatest in the cotton rats, especially to type 3 which gave titres ranging from <8 to 4096.

It is also evident that the degree of response to type 1 with the different species was similar, geometric mean titres of 53, 32 and 52 being obtained with monkeys, guinea-pigs and cotton rats respectively. In the case of type 2, however, monkeys gave the poorest response, guinea-pigs were slightly better and cotton rats better still, the mean titres being 60, 126 and 246 respectively. A more striking difference between the three species was shown in the response to type 3. With this type, monkeys and guinea-pigs responded poorly ; 15 of the 42 monkeys and 17 of the 40 guinea-pigs gave antibody titres of 8 or less, the mean titres being 24 and 11 respectively. Cotton rats on the other hand responded well to type 3 ; in only 3 animals was antibody not detectable and the mean titre was 112.

Antibody Response in Guinea-pigs to Two Doses

It might be concluded from the above results that of the species tested the cotton rat was the most suitable animal for studying the antigenic activity of poliomyelitis vaccine since it was able to give as good antibody responses to types

TABLE II.—*Antibody Titres of Sera from Monkeys, Guinea-pigs and Cotton rats after Immunization with Poliomyelitis Vaccine*

Species of animal	Virus type	No. of sera	Number of sera with antibody titre of :—																				Geo-metric mean titre
			<8	8	12	16	24	32	48	64	96	128	192	256	384	512	768	1024	1536	2048	3072	4096	
Monkey	1	43	2	0	5	1	7	1	6	6	4	2	2	3	3	1	0	0	0	0	0	0	53
	2	42	1	1	3	3	2	5	4	3	6	7	2	2	0	1	2	0	0	0	0	0	60
	3	42	10	5	4	3	1	1	3	1	1	1	2	0	5	2	0	3	0	0	0	0	24
Guinea-pig	1	40	4	2	—	10	—	8	—	5	—	4	—	7	—	0	—	0	—	0	—	0	32
	2	40	0	1	—	2	—	3	—	10	—	8	—	9	—	5	—	2	—	0	—	0	126
	3	40	7	10	—	14	—	5	—	3	—	1	—	0	—	0	—	0	—	0	—	0	11
Cotton rat	1	39	3	2	—	3	—	7	—	12	—	4	—	5	—	2	—	0	—	1	—	0	52
	2	35	1	0	—	1	—	2	—	4	—	2	—	11	—	7	—	2	—	5	—	0	246
	3	37	3	0	—	5	—	0	—	5	—	10	—	4	—	4	—	3	—	2	—	1	112

1 and 2 as the monkey or the guinea-pig and a decidedly better response to type 3 than either of these animals. It was considered, however, that the poor response of guinea-pigs to type 3 might be related to the immunization schedule and experiments were made to investigate the response to two doses of vaccine given at different intervals.

For this study vaccine batch E/2769 was used in groups of 14 or 15 guinea-pigs and the results are given in Table III. It is quite clear that the interval between the doses plays a most important part in stimulating the production of antibody. With type 1 no better response was obtained with the two doses separated either by an interval of 1 or 2 weeks than was obtained with only one dose of vaccine. There was a decided improvement in the response when the interval was extended to three weeks, a further improvement when it was extended to four weeks, the mean titre being 147, and this improvement was maintained with the six-week interval. Similar results were obtained with types 2 and 3, the mean titres with the four-week interval being 91 and 122 respectively.

TABLE III.—*Antibody Titres of Sera from Guinea-pigs after Immunization with Poliomyelitis Vaccine*

Virus type	No. of doses	Interval between doses (weeks)	No. of sera	No. of sera with antibody titre of :—										Geometric mean titre
				<8	8	16	32	64	128	256	512	1024	2048	
1	1	—	15	3	3	3	1	3	1	1	0	0	0	13
	2	1	15	1	6	4	2	2	0	0	0	0	0	13
		2	15	3	1	6	3	0	1	1	0	0	0	14
		3	15	1	0	2	4	3	3	1	0	1	0	51
		4	15	1	0	0	1	4	1	2	4	2	0	147
		6	14	0	0	2	0	2	3	1	3	3	0	190
2	1	—	15	5	4	3	1	1	1	0	0	0	0	8
	2	1	14	0	1	4	4	3	2	0	0	0	0	34
		2	14	3	3	3	1	4	0	0	0	0	0	12
		3	15	2	0	2	2	2	1	3	2	1	0	61
		4	14	0	0	1	4	4	1	2	1	0	1	91
		6	15	0	2	1	4	3	0	3	1	1	0	91
3	1	—	15	2	3	1	4	2	2	0	0	1	0	24
	2	1	15	1	2	2	5	2	3	0	0	0	0	28
		2	15	3	0	6	3	2	0	1	0	0	0	15
		3	15	1	0	0	3	6	4	0	0	1	0	61
		4	15	0	0	1	0	6	3	3	1	1	0	122
		6	14	1	0	2	1	4	1	2	2	1	0	78

CONCLUSIONS

The three batches of poliomyelitis vaccine used in the 1956 immunization programme showed similar antigenic activity when tested either in monkeys, guinea-pigs or cotton rats. A batch of American vaccine tested at the same time in all three species gave an activity of the same order as the British batches. These results reflect those obtained in the field study in which the responses of children, with initially no antibodies to any of the three types of virus, were found to be similar with the three batches of British vaccine (Medical Research Council Report, 1957). It is therefore probable that the guinea-pig or cotton rat may be used in place of the monkey in the laboratory control of the antigenic potency of

poliomyelitis vaccine. It is possible that guinea-pigs may prove more suitable since they are more easily handled and housed and give a greater volume of serum for testing than do cotton rats.

If guinea-pigs were to be used in the control testing, then consideration must be given to the immunization schedule. It is suggested, as a result of the experiments reported here, that two doses of vaccine at an interval of four weeks might be the best schedule to adopt since this gives maximum antibody responses to all three types of virus. Moreover, it would seem reasonable to use such a schedule since it is similar to the one used in the immunization of man.

The most suitable time to test antibody levels after immunization and the response to graded doses of vaccine are being investigated.

I wish to thank the Ministry of Health and Eli Lilly and Company for the supply of poliomyelitis vaccine.

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THE MODE OF ATTACHMENT OF A TYPHOID BACTERIOPHAGE TO THE CELL WALL OF ITS HOST

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MANY varieties of bacteriophage have been shown by electron-microscopy to consist of characteristic tadpole-shaped particles with well defined "heads" and "tails". These particles were first observed in dysentery and proteus phage lysates by Ruska (1941) who also noted that some of them could be seen adsorbed tail-first to their host cells. The published photographs are however not clear enough to show this. More convincing evidence that phage particles adsorb by their tails was obtained for coli phages T2 and T4 by Anderson (1952) using stereoscopic electron-microscopy and the critical point method of specimen preparation. His photographs show clearly among the many particles clustered around the cells several adsorbed by their tails. Penso (1952) showed photographs of phage particles adsorbed to mycobacteria in a similar fashion. Perhaps the clearest picture of adsorbed phage is that of Hotchin, Dawson and Elford (1952) who used a staphylococcal phage and the isolated cell walls of the host bacteria. Five particles can be seen adsorbed tail-first to one cell wall. However, the number of instances in which phages have been shown to adsorb in this manner is still very limited. It was therefore decided to examine a typhoid phage now in use in this laboratory and to determine its mode of adsorption. This paper describes the procedures used and the results obtained when this phage is allowed to interact with the isolated cell walls of its host *Salmonella typhi* O 901 R. The tail-first mode of adsorption is again evident and the tryptophan adsorption requirement of the phage is visually confirmed.

MATERIALS AND METHODS

The phage (phage 3) was obtained from Oxford sewage by subculture on *Salm. typhi* strain F1 in nutrient broth (Fildes and Kay, 1957). It requires tryptophan as an adsorption cofactor and is the only example of this peculiarity known to the authors outside the coli "T" group of phages of which T4 and T6 require tryptophan (Anderson, 1945). Phage stocks for the adsorption experiments were prepared on *Salm. typhi* O 901 R, a non-flagellated variant, growing in an ammonia-glucose medium supplemented with DL-tryptophan (0·001 M) and L-cystine (0·001 M) which are growth factors for this organism. This amount of tryptophan is also sufficient for the adsorption of the phage. After lysis of the culture the phage was purified by three cycles of low and high speed centrifugation and resuspended in phosphate buffer pH 7·6 (0·033 M) containing serum albumin (0·01 per cent).

Bacterial cell walls were prepared from *Salm. typhi* O 901 R grown to 1×10^9 cells/ml. in peptone water (2 per cent) agitated at 37° in a whirling-flask apparatus (Mitchell, 1949). The bacteria were collected, washed in water by centrifugation and crushed by shaking with fine glass beads in a Mickle shaker for one minute. This procedure damaged all the cells sufficiently to allow the cytoplasm to be released and to leave the majority of the cell walls intact. They were then washed in water by centrifugation until they appeared, in the electron microscope, to be largely free from fine particles.

Adsorption of Phage 3 to Cell Walls

A typical adsorption experiment was performed as follows. Cell walls suitably diluted in water (0·5 ml.) were added to phosphate buffer pH 7·6 (0·066 M, 0·5 ml.) containing DL-tryptophan (0·001 M). The buffer was added because phage adsorption does not take place in media of low ionic strength. Phage 3 (0·1 ml. containing 1×10^{10} particles) was then added and the mixture kept at 37° for 10 min. It was known from previous experiments that at least 95 per cent of the phage would be adsorbed in that time. To remove the unadsorbed phage the cell walls with adsorbed phage were washed once in buffer containing tryptophan and then fixed by the addition of formaldehyde (5 per cent final) for 10 min. at room temperature. After fixation the cell walls could be washed free of buffer without loss of adsorbed phage. Droplets of this aqueous suspension were then allowed to dry down on collodion-covered grids. Phage 3 alone cannot be suspended in water in the unfixed state without damage. It was therefore placed in the grids, fixed in the vapour of 40 per cent formaldehyde for 10 min. and then washed in water to remove salts. All specimens were shadowed with palladium.

RESULTS

Morphology of Phage 3

Fig. 1 shows that the particles of this phage possess the " head " and " tail " structure typical of many other phages. They resemble the coli phages T2, T4 and T6 (Williams and Fraser, 1953) in that the tail is terminated by a " knob " and the head is prismatic in shape. The dimensions taken from the photographs show the head to be 80 mμ wide and 100 mμ long. The tail is 115 mμ long, including the knob.

Mode of Attachment of Phage 3 to Cell Walls

Fig. 2 shows a typical field of phage-infected cell walls. Many particles can be seen attached to a long cell wall and to a mass of cell wall fragments by their tails with their heads pointing away from the walls. No phages appear to be adsorbed head first. All the other photographs taken show the same effect and support the view that the tail of this phage contains the specific organ of attachment.

Tryptophan Requirement for Adsorption of Phage 3

Confirmation of the requirement of tryptophan for adsorption of phage 3 as determined by the biological method (Fildes and Kay, 1957) is given in Fig. 3 and 4 which show the result of omitting tryptophan from an otherwise complete adsorption medium. The cell walls in both were mixed with phage, fixed and washed in the same way. Fig. 3 shows several adsorbed phages whereas in Fig. 4 the cell walls did not receive tryptophan and are devoid of phage particles.

DISCUSSION

Cell walls were chosen to attempt to demonstrate the adsorption of phage 3 by its tail for the following reasons. Attachment by the tail is only likely to be observed when a phage particle lies very near to the edge of a cell and when the " head " can be supported on the collodion film. If whole cells were used they would stand well above the surface of the film, even when dehydrated in the vacuum, and consequently a phage particle attached to the periphery, as viewed from above, would take up a position with its " head " against the wall so obscuring the point of attachment. On the other hand cell walls are very thin

and lie close to the film ; consequently a phage particle adsorbed tail-first to the periphery would be supported with its head away from the wall. The photographs show that under conditions of ionic strength and temperature in which the phage would adsorb to its living host cell the mode of attachment is by the tail as described by Anderson (1952) for the coli phages. This observation supports the general view that all phages adsorb by their tails. Further evidence obtained with other phages would still be welcome because phage 3 is in several ways very much like the coli phages used by Anderson. These similarities are in morphology (Putnam, 1950), the tryptophan requirement already mentioned and the common possession of the unusual pyrimidine 5-hydroxymethyl cystosine (Wyatt and Cohen, 1952 ; Kay, 1957, to be published). The host range of the typhoid phage is however quite different from the coli phages and so far no common host has been found. An unexpected feature of the photographs showing adsorbed phage is that the particles do not appear to have flattened heads and presumably have not released their nucleic acid. This is also a feature of the photographs of Penso (1952) and of Hotchin *et al.* (1952) although in the latter case some of the phage heads are clearly deflated. Perhaps adsorption to isolated cell walls is insufficient to cause the release of phage nucleic acid, intact cells being necessary for this to happen.

SUMMARY

The morphology of a tryptophan-dependent typhoid phage is described and its mode of attachment to the cell walls of its host bacterium examined. It is found to adsorb tail first. The tryptophan adsorption requirement is visually confirmed.

The authors wish to acknowledge the assistance and valuable criticism given by Sir Paul Fildes, F.R.S., Director of the Unit.

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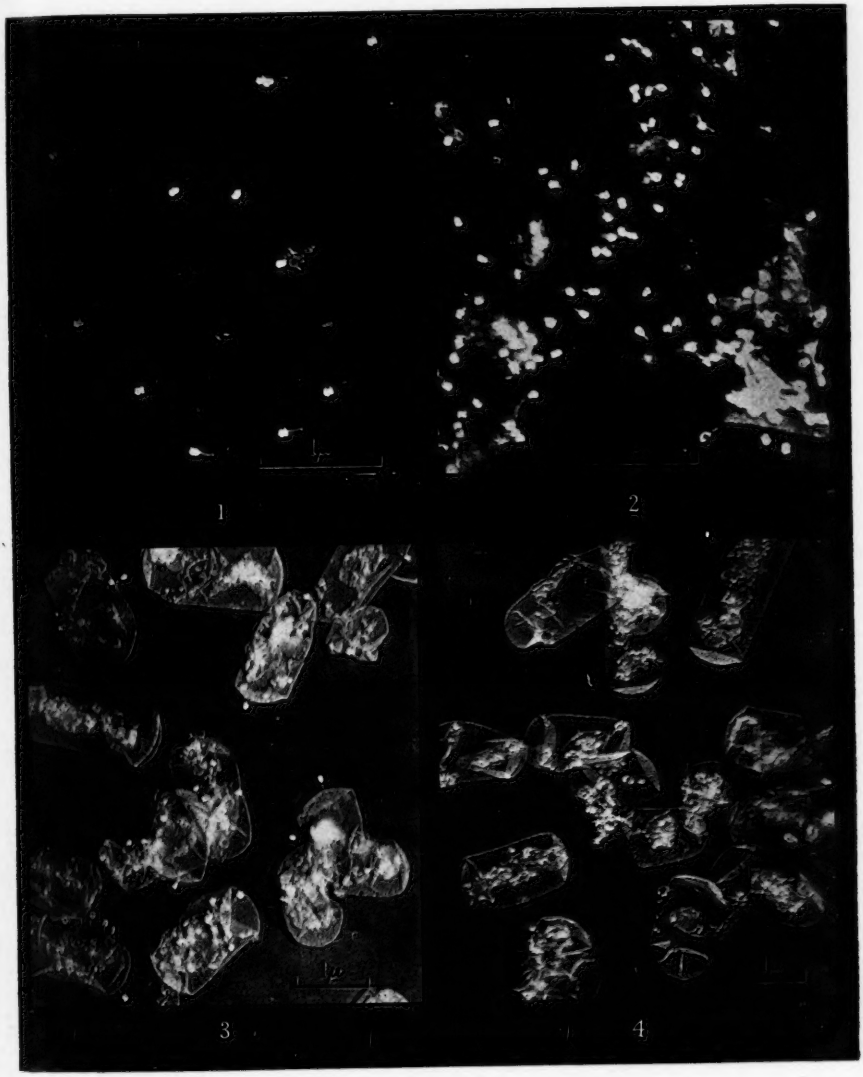
EXPLANATION OF PLATES

Fig. 1.—Purified specimen of phage 3.
Fig. 2.—Phage 3 adsorbed to cell walls of *Salm. typhi* O 901 R.
Fig. 3 and 4.—Interaction of phage 3 and *Salm. typhi* O 901 R cell walls with (Fig. 3) and without (Fig. 4) tryptophan.



Kay and Sampson.

STUDIES ON THE PRODUCTION, MODE OF ACTION AND PROPERTIES OF INTERFERON

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THE phenomenon of viral interference has been studied extensively in the past twenty years, and particular attention has been paid to the inhibitory effect on influenza virus growth produced by previous inoculation with killed virus of either the same strain or a different serological type.

In a previous paper (Isaacs and Lindenmann, 1957) we described the occurrence of a product of influenza viral interference which we called "interferon". Interferon was produced by the interaction of heat-inactivated influenza virus and chick chorio-allantoic membranes *in vitro*, and its presence was revealed by its ability to induce interference in fresh pieces of chorio-allantoic membrane. It was also shown that interferon could be distinguished from heat-inactivated influenza virus by several properties; thus interferon was non-haemagglutinating, its activity was not neutralized by viral antiserum, it was not sedimented by centrifugation at 100,000*g* and surprisingly, it was held up by filtration through gradocol membranes of average pore diameter 0·5–1 μ. However, it was not possible to decide whether interferon was a cellular product formed as a reaction to the virus, a part of the heated virus itself, or whether it represented an abortive attempt at virus multiplication. In the present paper we describe further experiments relating to the production, mode of action and properties of interferon.

MATERIALS AND METHODS

Interfering virus.—In most experiments the Melbourne strain of influenza virus A was used as freshly harvested allantoic fluid, mixed with a 2 per cent sodium citrate solution in normal saline and borate buffer pH 8·5, in the ratio 6 parts virus, 2 parts citrate-saline and 1 part borate buffer. This was heated at 56° for 1 hr., which abolishes the infectivity of the virus while retaining its interfering activity. The heated virus is referred to as heated MEL.

In a few experiments the virus was inactivated with ultra-violet light. It was first partially purified by absorption from infected allantoic fluid on to chick red cells at 0° and elution at 37° into normal saline. The eluate was then irradiated in shallow layers with a mercury vapour germicidal lamp giving maximal emission at 2537 Å. The time for irradiation was decided empirically by using double the minimal time necessary to inactivate the infectivity of the virus preparations used.

Other virus strains.—Other viruses used were the PR8 strain of influenza A, the haemagglutinating virus of Japan or Sendai virus (Kuroya, Ishida and Shiratori, 1953) and vaccinia virus. All strains were kept in capillary tubes at − 70°.

Buffers.—The buffer which we prefer is that described by Earle (see Parker, 1950), but at one point in these studies a bicarbonate buffer could not be used and a glucosol-phosphate buffer was used instead (see Fulton and Armitage, 1951).

Chorio-allantoic membranes.—Membranes from 10– or 11-day eggs were used. If whole membranes were required the shell was removed from the pointed end of the eggs, the egg

contents poured out and the membranes were then removed and washed in two changes of buffer. When small pieces of membrane were required pieces of shell with membrane attached were cut out of the de-embryonated egg, and the membrane pieces were then detached and washed. Six or seven pieces were taken from each egg and membranes were pooled from a group of eggs and randomised.

Interference and challenge

An interference experiment was usually carried out as follows : small pieces of membrane were placed in $6 \times \frac{5}{8}$ in. ($15 \times 1{\cdot}5$ cm.) test tubes, 1 piece in each tube along with 1 ml. of test or control material. One hundred units of penicillin were added per ml. of fluid and six tubes were used for each experimental group. The tubes were stoppered and placed in a roller drum at 37° (8 rev./hr.). After 18–24 hr. incubation the fluids were removed and replaced with 1 ml. buffer in which MEL virus at a dilution of 10^{-3} was incorporated. In some experiments the membranes were washed before challenge with MEL but usually this step was not necessary. The tubes were then placed in the roller drum for a further 48 hr. at 37° after which the fluids were titrated individually for their haemagglutinin content. A 48 hr. challenge period gave more satisfactory results than a 24 hr. period. Where this procedure was varied it is indicated in the text.

Haemagglutinin titrations

Two-fold dilutions (0·25 ml.) of test virus were made in normal saline using automatic pipettes and plastic plates. To each dilution 0·25 ml. of a 0·5 per cent suspension of chick red blood cells was added, and the cells allowed to settle. That pattern which showed partial agglutination was taken as the end-point, and it was read by interpolation if necessary. One agglutinating dose (A.D.) is defined as the amount of virus present at the partial agglutination end-point. The readings in the tables are given as $\log_2$, *i.e.*, tube number of a series of two-fold dilutions. Thus if the end-point of agglutination occurs at a 1/2 dilution the reading is taken as $\log_2 = 1$. If the end-point of agglutination occurs at a 1/4 dilution the reading is taken as $\log_2 = 2$. A 1/1 dilution end-point has a $\log_2 = 0$ and material which did not agglutinate red cells at a 1/1 dilution was given an arbitrary score of -1.

Assessment of results

The results are given in the tables as geometric mean haemagglutinin titres of a group of six fluids, the titres being expressed as the $\log_2$ of the dilution at the haemagglutination end-point. The tables also show the haemagglutinin yield in each group expressed as the per cent of the corresponding control. Evidence has previously been given (Isaacs and Lindenmann, 1957) that if an experimental group of six tubes showed a geometric mean titre of one $\log_2$ unit less than a group of six controls tested at the same time this indicated a slight but statistically significant degree of interference, and a difference of two $\log_2$ units indicated definite interference. Such differences correspond to a 50 per cent yield and a 25 per cent yield compared with the controls, and small arithmetic differences in the percentage yield have a much greater significance at low than at high percentage yields.

By making use of the observation that a linear relation exists between the concentration of interferon and the degree to which the virus yield is depressed (Isaacs, Lindenmann and Valentine, 1957) it was possible to make a rough comparison of the potency of different preparations.

Pressure dialysis.—Interferon was concentrated by pressure dialysis. Fluids were placed in large cellophane Visking sacs attached to a glass bulb as reservoir, and immersed in a cylinder of buffer and compressed air was forced into the bulb to a pressure of 600 mm. mercury. The sacs were left for 48 hr. at 2° when the volume had usually decreased to about 1/10th. Some insoluble material usually precipitated out but there was no significant loss of interferon activity.

Enzymes.—Trypsin : a crystalline preparation from Armour and Co. Ribonuclease was kindly supplied by our colleague Dr. R. R. Porter. Crystalline deoxyribonuclease : the Worthington Biochemical Co. Receptor-destroying enzyme of *V. cholerae* (RDE) : a crude extract was obtained from Phillips-Roxane Co. and from this the enzyme was partially purified by absorption on chick red cells at 0° and elution into saline (Burnet and Stone, 1947). As titrated by the method of Burnet and Stone eluates usually gave a titre of about 640 units/ml.

Miscellaneous.—In experiments involving precipitation of interferon with ammonium sulphate or addition of sodium periodate or urea, it was necessary to remove these reagents before interferon could be tested biologically. Small-molecular substances such as these were removed by dialysis against large volumes of buffer at 2°, usually with one or two changes of buffer at daily intervals.

RESULTS

Production of Interferon

In previous experiments interferon was produced in small amounts in test tubes. For the larger yields required for isolation and characterisation of interferon it was necessary to simplify and standardise its production. At the same time the effect of variables on the yield was studied.

Production in bottles.—Roux bottles of capacity 1 l. were found convenient. Entire chorio-allantoic membranes were removed from 10- or 11-day chick embryos, washed in buffer and 10 membranes placed in a Roux bottle with 50 ml. of suitably diluted heated MEL virus. Such bottles were then placed at 37° in a horizontal shaking machine delivering 86 strokes/min. (to-and-fro) with an amplitude of about 1 in. (2·5 cm.). After 3 hr. incubation the membranes were removed, washed twice in buffer and re-suspended in 50 ml. volumes of buffer in fresh bottles. These were returned to the shaking machine and incubated at 37° overnight. The fluid was then removed and centrifuged at about 1000 **g** for 20 min. and the supernatant fluid used as a standard interferon preparation.

Dosage of heated virus.—In most experiments MEL virus was heated at 56° for 1 hr. in citrate-borate buffer (Methods) and used at a dose of 5 ml. undiluted virus per membrane. However, in experiments to test the effect of virus dosage on yield of interferon it was found that the yield of interferon with 5 ml. of a 1/10 dilution of virus per membrane was almost as good as the yield with undiluted virus. With virus diluted 1/100 there was a sharp decrease in yield. With virus diluted 1/10, the amount of interfering activity produced by the membranes as interferon was about the same as that which the heated virus itself would show in a direct interference test. Fazekas de St. Groth and Edney (1952) noted that the degree of interference produced by heated influenza virus (Lee strain) was maximal when there were roughly 500 agglutinating doses of virus per egg. In the present experiments the maximal yield of interferon occurred when about 800 agglutinating doses of virus per membrane were used.

Conditions of incubation.—When virus-treated membranes were suspended in 50 ml., 100 ml. and 200 ml. of buffer it was found that the yield of interferon in a bottle containing 200 ml. was considerably less than might be expected on the basis of dilution. The large volume increases the violence of agitation but we have not investigated what bearing this factor may have on the yield. However it was found that a bottle containing 50 ml. of buffer kept stationary at 37° gave roughly half the yield of interferon of a similar bottle kept in the shaking machine, so that aeration may play an important part in the yield.

It seemed likely that some metabolic activity was required from the membranes for interferon to be produced. If this were so, one would expect that incubating membranes at 2° instead of 37° would greatly diminish the yield. This was verified in the following experiment. Six chorio-allantoic membranes were incubated with 30 ml. heated MEL for 3 hr. at 37° in the shaking machine. The membranes were then thoroughly washed and incubated without shaking, with

20 ml. buffer, 3 at 37° and 3 at 2°. After overnight incubation the fluids were harvested and tested for their interferon content. The results are shown in Table I.

TABLE I.—*Titration of Interferon from Membranes Incubated at 2° and 37°*

Number	Individual haemagglutinin titres of		
	Interferon 2°	Interferon 37°	Buffer control
1	6	0·5	5·25
2	4	<0	6
3	6	<0	6·25
4	4·5	<0	5
5	4·5	<0	5·25
6	4·5	<0	5·25
Average per cent of control yield	66	<3	100

Haemagglutinin titres are expressed as $\log_2$ of dilution end-point.

Chorionic and allantoic surfaces.—It is known that the chorionic and allantoic surfaces of the membranes differ in their production of influenza virus and in their behaviour as regards viral interference (Isaacs and Fulton, 1953). It was therefore of interest to see whether application of heated virus to either surface of the membrane would lead to production of interferon.

Groups of 10-day eggs were inoculated with 1 ml. of heated MEL virus per egg (*a*) by the allantoic route and (*b*) on the dropped chorionic surface, and in addition a control group of membranes was mixed with the same amount of virus *in vitro* in the standard manner. After 3 hr. incubation the eggs were opened, the membranes removed and washed thoroughly and membranes from all three groups were incubated with buffer overnight at 37°. It was found that interferon of high and comparable activity was produced in all three groups.

Second cycle of interferon production.—It has been previously observed (Isaacs and Lindenmann, 1957) that following incubation of membranes with heated influenza virus the bulk of interferon was liberated between the 3rd and 12th hours. In agreement with this, it was found that whole membranes which had produced interferon in the standard manner when re-suspended in fresh buffer for a second 24 hr. period at 37° did not release more interferon. The possibility was considered that the membranes might have been exhausted of some constituent necessary for the production of interferon. To test this, whole membranes which had liberated a first crop of interferon were mixed with the same dose of heated influenza virus a second time. It was found that these membranes liberated a second crop of interferon which was almost as potent as the first. Such membranes would be expected to show interference on challenge with living virus and it seemed important, therefore, to establish whether production of good yields of interferon was compatible with a highly significant degree of interference. This was tested in the following experiment.

Small pieces of chorio-allantoic membrane were incubated with heated MEL, one piece with 1 ml. of virus per tube. After 3 hr. at 37° the membranes were washed and incubated in 1 ml. amounts of buffer overnight at 37°. Controls received buffer only. After overnight incubation 6 membranes were tested by challenge with MEL virus to see what degree of interference had been established

and it was found that the yield in these membranes was roughly 3 per cent of that of control membranes, *i.e.*, 97 per cent interference was established. At the same time some fluid from these membranes was harvested and it was found that interferon had been liberated. Further groups of these membranes were then incubated a second time with either heated MEL or buffer in the same way, to see whether a second crop of interferon would be produced. The results are shown in Table II.

TABLE II.—*Production of a Second Crop of Interferon*

Group number	1st inoculation	2nd inoculation	HA titre*	Per cent of control yield
1	Heated MEL	Buffer	6·0	71
2	Buffer	Heated MEL	2·0	4·5
3	Heated MEL	Heated MEL	2·9	8·5
4	Buffer	Buffer	6·5	100

* Expressed as mean $\log_2$ of dilution end-point.

The results with group 1 in Table II show that membranes which had produced interferon in the first 24 hr. period did not do so when incubated with buffer in the second 24 hr. period. From group 3 it can be seen that a second inoculation of heated MEL led to just over half the amount of interferon produced in membranes previously treated with buffer (group 2). This yield is out of all proportion to the degree of interference established and it is concluded that production of interferon can occur in membranes in which a high degree of interference with viral synthesis has been produced.

Production of interferon by virus inactivated with U.V. light.—In most experiments we have prepared interferon with virus inactivated by heat, which is a technically simple procedure with large volumes of virus. However, since it is well known that virus inactivated by ultra-violet light is a more efficient interfering agent than virus inactivated by heat (Henle, 1953) it was interesting to see how the two compared as regards interferon production.

In a single experiment in which MEL virus was inactivated by the two methods heated virus at a concentration of 5 ml. per membrane was compared with ultra-violet-inactivated virus at a fifth of this concentration. In spite of the difference in virus dosage the interferon from ultra-violet-inactivated virus was eight times more potent than the heated virus preparation. It was in fact the most potent preparation we had and it is planned to carry out future studies with interferon prepared in this way.

In further experiments the effect of different times of irradiation of the virus was investigated. The membranes used for production of interferon were challenged to establish the degree of interference, and the liberated interferon was titrated in each group. Irradiation for 1, 2 and 4 min. led to the establishment of a high degree of interference in the membranes, with corresponding high yields of interferon. Irradiation for 8 min. gave a significantly lower degree of interference in the membranes while no detectable interferon was produced. However, the test for membrane interference used was more sensitive than the test for the presence of interferon.

The results parallel earlier findings with heated virus (Isaacs and Lindenmann, 1957); virus heated at 56° initiated interference and caused production of interferon while virus heated at 60° did neither.

Action of Interferon

Time of challenge.—In the standard test procedure membranes were kept in contact with interferon for 24 hr. before challenge, a procedure which was based on the fact that interference due to heated influenza virus takes some hours to be established (Fazekas de St. Groth, Isaacs and Edney, 1952). It was found that when the challenge virus was applied along with interferon no interference resulted. In a further experiment in which the interval between introducing interferon and challenge virus was varied, it was found that only slight interference was present with an interval of 6 hr., but there was definite interference at 24 hr. which was not improved by increasing the interval to 48 hr.

Absorption of interferon.—The time interval required for interference to be established might be due to a slow rate of absorption of interferon by the membranes or to the need for some metabolic process lasting a few hours before interference became established. In fact some evidence has been found that both these processes may operate.

In order to measure absorption of interferon a number of tubes were set up each containing 1 ml. of a preparation of interferon and one small piece of chorio-allantoic membrane. These were incubated for 24 hr. at 37° in the roller drum and it was shown that interference was established in the membranes. The fluids were then removed and dialysed against buffer to restore the original salt and glucose concentrations and as a control an aliquot of the original interferon preparation was dialysed at the same time. The interfering activities of these two preparations were then tested ; a comparison between the two indicates the amount of interferon which was absorbed or used up during 24 hr. contact with the membranes. The results are stated in Table III under the heading " first absorption cycle ". It will be seen that the interfering activity was only slightly reduced by 24 hr. contact with the membranes. The experiment was then continued by repeating each experimental step but starting with the first cycle fluid, and in Table III the results are stated under the heading " second absorption cycle ". This shows that a further slight reduction of interfering activity occurred after the second absorption period, but a substantial amount of interfering activity remained unabsorbed. The findings suggest that in this system absorption of interferon by chick chorio-allantoic membranes is not an efficient process.

TABLE III.—*Absorption of Interferon by Living Membranes*

	Buffer control		Interferon after absorption		Interferon controls	
	HA*	Per cent†	HA	Per cent	HA	Per cent
1st absorption cycle 24 hr.	7·3	100	3·3	6	2·8	4
2nd absorption cycle 2 × 24 hr.	6·8	100	3·6	11	2·1	4

* mean $\log_2$ haemagglutinin titre of 6 tubes.
† per cent control titre.

Effect of temperature on establishment of interference.—The finding that interferon was inefficiently absorbed was surprising since we had earlier found that during a 4 hr. period of contact between interferon and membrane, followed by

20 hr. incubation at 37°, satisfactory interference resulted. However, it was now possible to separate the period of absorption of interferon from its period of action and to test the temperature requirements for the latter.

Four groups of membranes were incubated at 37°, one with buffer and three with interferon. After 3 hr. two of the interferon groups were removed, the membranes washed thoroughly and suspended in buffer. One of these groups was incubated at 37° for 21 hr. and the other group at 2°. The remaining groups were kept for the whole 24 hr. at 37°. At the end of this period, all four groups were challenged with influenza virus in the usual way. The results are shown in Table IV.

TABLE IV.—*The Effect of Temperature on the Establishment of Interference by Interferon*

Group number	Experimental group	HA*	Per cent of control yield
1	Control—buffer	6·9	100
2	Interferon—24 hr. 37°	0·3	1
3	Interferon—3 hr. 37°—Buffer 21 hr. 2°	5·6	41
4	Interferon—3 hr. 37°—Buffer 21 hr. 37°	2·4	4

* Mean $\log_2$ of virus haemagglutinin titre.

The difference between the last groups shown in Table IV indicates that once interferon has been absorbed for a short period of time, further incubation at 37° is still necessary before interference is established.

These experiments on the effect of time of challenge and incubation temperature on the establishment of interference make it clear that interferon acts on the membrane and not on the extracellular virus. Furthermore no effect of interferon on the viral haemagglutinin could be demonstrated.

Dose of challenge virus.—Fazekas de St. Groth and Edney (1952) showed that with heated influenza virus as interfering agent the degree of interference produced was within limits independent of the dose of challenge virus, provided that adequate time was allowed for interference to be established before the challenge virus was introduced. Experiments were carried out with a single dose of interferon given to three groups of membranes and buffer in three control groups. After 24 hr. incubation pairs of interferon and control groups were challenged with a 10^{-3} dilution of MEL virus in the standard way, while other groups received virus diluted 10^{-4} and 10^{-5}. No significant differences were found in the degree of interference in the different groups.

Specificity of interferon.—Attempts were made to prepare interferon from heat-inactivated influenza virus of strains other than MEL, but with virus heated at 56° for 1 hr. the results were very variable. Interferon was therefore prepared from MEL and PR8 strains of influenza virus by ultra-violet irradiation for a standard time and tested for its activity against three different challenge viruses. The results are shown in Table V. The interferon prepared from PR8 was less active than that prepared from MEL when tested against all three strains of challenge virus. Also, Sendai and PR8 viruses were found to be slightly more sensitive to interferon when tested with both preparations. Thus no evidence of specificity of action of interferon was found.

TABLE V.—*Absence of Specificity in Action of Interferon*

Challenge virus	Yield measured as	Interferon from MEL	Interferon from PR8	Interferon from *Nil* (control)
MEL	HA titre*	2·4	3·2	6·9
	Per cent of control yield	4·5	7·7	100
PR8	HA titre*	0·5	2·5	6·8
	Per cent of control yield	1·3	5·2	100
Sendai	HA titre*	−1·0	2·2	6·1
	Per cent of control yield	<1·5	6·7	100

* Haemagglutinin titre expressed as mean $\log_2$ of a group of 6 tubes.

Effect of interferon in eggs and rabbits.—Interferon is known to interfere with the growth of vaccinia virus in membrane fragments *in vitro* (Isaacs *et al.*, 1957). In order to test whether interferon would act *in ovo*, we prepared a twenty-fold concentrate of interferon by pressure dialysis (see below) and applied 0·1 ml. volumes of this concentrate on dropped chorionic membranes of 11-day eggs. After 24 hr. the eggs were challenged by chorionic inoculation of suitably diluted vaccinia virus and the number of pocks appearing after 48 hr. further incubation was compared with controls. The interference thus established was barely significant. Attempts to produce interference by injecting interferon into the allantoic cavity and challenging with influenza and related viruses likewise failed to show interference. But these experiments will be repeated since, because of the large amounts of interferon needed, we did not try to maintain in the whole egg a relationship between the amount of interferon and the tissue mass comparable to that obtained *in vitro*. When the interferon concentrate was diluted to original volume in normal allantoic fluid instead of buffer and tested by the usual system *in vitro* the expected amount of interference resulted; thus failure of interferon to act in eggs could not be attributed to its inactivation by some component of normal allantoic fluid.

Heated MEL injected intradermally into rabbits interferes locally with the subsequent development of vaccinial lesions (Depoux and Isaacs, 1954). The same effect could be produced by the intradermal injection of 0·1 ml. of a mixture of ×20 concentrated interferon and a 10^{-5} dilution of vaccinia virus. If the challenge with vaccinia was delayed 24 hr. the effect was less obvious, but this may be due to the difficulty of giving the second injection at exactly the same place as the first. *In vivo* experiments are being continued with more concentrated preparations.

Some Properties of Interferon

Pressure dialysis and freeze-drying.—We had previously found that interferon could be dialysed against buffer without loss of activity (Isaacs *et al.*, 1957). Pressure dialysis proved a convenient way of concentrating interferon up to 10- or 20-fold. When made up with buffer to original volume, such preparations showed no loss of activity. Attempts at a similar concentration by freeze-drying failed. When standard interferon preparations were first dialysed against distilled water, then freeze-dried and made up to volume with buffer, considerable activity could be recovered, but it proved impossible to take up the whole freeze-dried material in small volumes of buffer, since an insoluble precipitate formed which retained substantial amounts of interfering activity.

pH stability.—The pH stability of interferon was tested by dialysing at 2° against large volumes of buffer over the pH range 1–10. After 24 hr. at 2° at the appropriate pH the preparations were dialysed against Earle's solution in order to restore them to their original pH and then tested for their interfering activity. Interferon was stable over the entire pH range.

Treatment with urea.—Attempts were made to dissolve the residue obtained by freeze-drying interferon, previously dialysed against water, in small volumes (1/25th original) of 6M urea. This gave only about a 30 per cent recovery of interferon. It is known that solution of some proteins in 6M urea leads to denaturation sometimes due to molecular dissociation or an increase in molecular asymmetry (Putnam, 1953). However, interferon was stable in 6 M urea and filtration through gradocol membranes showed that the filtration behaviour had not altered (Isaacs *et al.*, 1957). Further, ultra-sonic treatment of interferon (six $\frac{1}{2}$ min. bursts of 350 k.c.) caused no change in filtration behaviour; *i.e.*, complete activity was recovered after filtration through a membrane of average pore diameter 1·8 μ and partial loss of activity occurred with a 1·0 μ membrane.

Miscellaneous.—Interferon was precipitated from solution by saturation with ammonium sulphate and full activity was recovered after addition of the original volume of buffer to the precipitate. Treatment of an interferon preparation (previously dialysed against saline) with sodium periodate ($NaIO_4$) at a final concentration of 0·001, 0·0001 and 0·00001M for 1 hr. at room temperature followed by dialysis against buffer caused no decrease in activity. Shaking with an equal volume of ether at room temperature caused complete loss of activity from ether and aqueous fractions.

Trypsin.—A preparation of interferon was incubated for 2 hr. at 37° with crystalline trypsin at a concentration of 0·01 per cent in Earle's buffered saline. Its interfering activity was greatly reduced by this treatment, and even incubation with 0·001 per cent trypsin considerably reduced its activity, but complete loss of activity was not found.

It seemed possible that the trypsin might have been acting on the membrane rather than the interferon. In order to decide on the site of action of the enzyme, soya bean inhibitor was added (*a*) along with the trypsin (*b*) after incubating interferon with trypsin and (*c*) in a control group without trypsin. The results of an experiment with 0·025 per cent trypsin and 0·025 per cent soya bean inhibitor are shown in Table VI.

TABLE VI.—*Effect of Trypsin on Interferon Action*

Group number	Material tested	$\log_2$ HA titre	Per cent of control yield
1	Interferon control	<0	<1·6
2	Interferon incubated with trypsin	3·25	15
3	Interferon incubated with trypsin followed by soya bean inhibitor	3·3	15
4	Interferon incubated with soya bean inhibitor mixed with trypsin	<0	<1·6
5	Interferon incubated with soya bean inhibitor	<0	<1·6
6	Buffer control	6·0	100

Table VI shows that the trypsin alone partly inactivated interferon, that the effect of the trypsin was completely blocked by mixing it with soya bean inhibitor (group 4) but that the soya bean had no action when it was added to the system after interferon had been incubated with trypsin, but before the chorio-allantoic membranes were added. This shows that the trypsin effect was due to its acting directly on the interferon and not on the membrane.

Other enzymes.—It was difficult to test the effect of various enzymes on interferon unless they could be readily removed from the system or their action inhibited before the membranes were added. The alternative was to incubate interferon for 2 hr. at 37° with low concentrations of enzymes and to use the same concentrations mixed with buffer in the controls. When ribonuclease (0·001 per cent), deoxyribonuclease (0·001 per cent) and the receptor-destroying enzyme of *V. cholerae* (RDE) were used in this way no definite effect on interferon could be demonstrated. In one experiment interferon was incubated with 0·025 per cent trypsin for 2 hr. at 37°, 0·025 per cent soya bean inhibitor was added and it was then incubated with 0·001 per cent ribonuclease for 1 hr. The results are shown in Table VII.

TABLE VII.—*Effect of Trypsin Followed by Ribonuclease on Interferon*

Group number	Material tested	$\log_2$ HA titre	Per cent of control yield
1	Interferon	1·6	2·9
2	Interferon incubated with trypsin	5·3	28
3	Interferon incubated with trypsin, followed by ribonuclease	4·3	14
4	Buffer control	6·7	100

It can be seen that trypsin partly inactivated the interferon preparation, and that the ribonuclease did not further inactivate it. The slight depression of virus yield seen in this and in control experiments is probably due to the action of residual ribonuclease on the membranes (LeClerc, 1956).

In one experiment a slight apparent effect of RDE on interferon was observed, but this could not be confirmed in a second experiment. One difficulty is that RDE itself inhibits influenza viral growth in this system and a second difficulty, pointed out by Dr. A. Gottschalk, is that RDE may show variable behaviour from experiment to experiment. In order to overcome these difficulties, interferon was incubated with large doses of live influenza virus (approximately 2500 agglutinating doses of MEL virus/ml. interferon) for 2 hr. at 37° and the virus was effectively removed from the system by addition of viral antiserum and high speed centrifugation followed by further addition of antiserum. Under these conditions no significant action of the viral enzyme on interferon was observed.

DISCUSSION

In its time of appearance following inoculation of virus, and in the presence of an " eclipse phase ", interferon production has shown interesting analogies with virus production (Isaacs and Lindenmann, 1957). However two differences have now emerged. First, interferon was produced in approximately the same

degree whether virus was inoculated on the allantoic or chorionic surfaces of the chorio-allantoic membrane, whereas it is known that the cells on these two surfaces differ considerably in their ability to support multiplication of influenza virus. Secondly, when a large dose of heated virus was incubated with chorio-allantoic membranes interferon was liberated and *interference* was induced, *i.e.*, the membranes showed a greatly reduced ability to support influenza virus multiplication. Nevertheless, these membranes were able to produce a second crop of interferon normally when inoculated with a second dose of heated virus. Both these findings suggest that the whole of the complex machinery required for viral synthesis is not required for production of interferon.

We have already noted that in different systems there was a rough parallel between the degree of interference with viral synthesis induced in membranes and the amount of interferon liberated. In the present study a further correspondence was found, particularly with virus inactivated by ultra-violet light for various periods of time. Interferon is very slowly taken up by chorio-allantoic membranes and incubation of these membranes for some hours at 37° is required before interference is established. This last finding is reminiscent of the fact that interference by heated virus takes some hours to be established even after the virus has been taken up by the cells. The action of interferon also resembles interference by heated virus in two further ways. The degree of interference does not depend on the dose of challenge virus and there is no strain specificity in the action of either agent. Thus although we have no direct evidence that interference is mediated through the action of interferon there is growing circumstantial evidence that this may be the case.

There is little new information on the nature of interferon. Its sensitivity to digestion with trypsin suggests that it may contain protein but there is insufficient evidence to allow any firm conclusions on the presence or absence of nucleic acids, carbohydrate or lipids in combination with the protein. It seems unlikely that we can learn much about its chemical nature until interferon can be prepared in purified form.

SUMMARY

Experimental conditions for preparing interferon in bulk are described. Interferon was produced in similar amounts when heated influenza virus was inoculated on either the allantoic or chorionic surfaces of the chick chorio-allantoic membrane.

Membranes which had produced interferon and which were shown to be unable to support the growth of influenza virus nevertheless produced a second crop of interferon when incubated a second time with heated influenza virus.

Influenza virus inactivated by ultra-violet light gave rise to more potent preparations of interferon than virus inactivated by heat. In these and in other experiments a correspondence was noted between the degree of interference induced and the amount of interferon liberated.

In addition to its action *in vitro*, interferon was found to inhibit the development of pocks by vaccinia virus in the rabbit skin.

Interferon was partly destroyed on incubation with crystalline trypsin.

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